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14. ABSTRACT

Defined by the presence of malignant cells spreading within intact prostatic ducts and acini, intraductal carcinoma of the prostate (IDC-P) occurs almost exclusively in high Gleason grade and stage tumors and is a consistent independent risk factor for tumor progression and death. Importantly, however, IDC-P is currently systematically under-diagnosed in needle biopsies because it has significant morphologic overlap with another intraepithelial lesion, high grade prostatic intraepithelial neoplasia (HGPIN). We hypothesize that IDC-P represents progression of invasive high-risk prostate carcinoma, and thus is molecularly distinct from HGPIN. Herein, we have begun to conduct an unbiased, three-pronged set of experiments to definitively identify the molecular signature of IDC-P using a combination of protein, RNA, and DNA-based assays. Aim 1 of the project has been completed following HRPO approval (5-22-15) and results were recently published. Currently, we are working to finish Aims 2 and 3.

15. SUBJECT TERMS
Prostate cancer, intraductal carcinoma, molecular profiling

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Table of Contents

Page

1. Introduction……4

2. Keywords……4

3. Overall Project Summary……...4-7

4. Key Research Accomplishments……7

5. Conclusion……7

6. Publications, Abstracts, and Presentations……..…….8

7. Inventions, Patents and Licenses……8

8. Reportable Outcomes..……8

9. Other Achievements……8

10. References……..8

11. Appendices…….9-54

12. Figures and Tables……55-57

1. INTRODUCTION:

Although non-invasive, intraductal carcinoma of the prostate (IDC-P) has long been recognized by pathologists as an extremely high risk feature. Defined by the presence of malignant cells spreading within intact prostatic ducts and acini, IDC-P occurs almost exclusively in high Gleason grade and stage tumors and is a consistent independent risk factor for tumor progression and death in cohorts treated with surgery or radiotherapy. Importantly, however, IDC-P is currently systematically under-diagnosed in needle biopsies because it has significant morphologic overlap with another intraepithelial lesion, high grade prostatic intraepithelial neoplasia (HGPIN). Since HGPIN is a morphologically similar lesion with virtually no prognostic significance, ***we propose that the systematic under-diagnosis of IDC-P in needle biopsies results in the under-recognition of potentially aggressive prostate tumors***. We have found that IDC-P and HGPIN may be readily separable at the *molecular* level, as IDC-P shows an extremely high rate of PTEN loss (84%), a rate even exceeding that seen in invasive high Gleason grade tumors. In contrast, HGPIN never shows loss of this tumor suppressor. Although our preliminary candidate gene data is compelling, *the current challenge is to systematically elucidate the molecular profile of IDC-P, a study which will not only yield additional clinically useful markers of this specific lesion but also elucidate the molecular features of an extremely high risk subset of prostate tumors*. The aims of the current study are to: 1) *Validate* PTEN and ERG as specific, clinically applicable markers of IDC-P, using a combination of immunohistochemistry and fluorescence *in situ* hybridization (FISH); 2) *Profile* the gene expression signature of IDC-P and systematically compare it to HGPIN, identifying additional candidate markers for distinguishing the two lesions; and 3) *Integrate* IDC-P into the molecular landscape invasive carcinoma, both at the gene expression and genomic levels, using a combination of bioinformatics, targeted next generation sequencing and copy number variation analysis.

2. KEYWORDS:

KEYWORDS: Prostatic carcinoma, intraductal carcinoma, high grade prostatic intraepithelial neoplasia, molecular profiling

3. OVERALL PROJECT SUMMARY:

Task 1: *Validate* PTEN and ERG as specific molecular markers of IDC-P (months 4-24, allowing for 3 month regulatory review of IRB protocols)

1a. Assess PTEN/ERG protein status via immunohistochemistry (IHC) in 40 biopsies each of: isolated IDC-P meeting current morphologic criteria, IDC-P with concurrent invasive carcinoma, and age-matched cases of isolated HGPIN (months 4-10)

1b. Assess whether PTEN protein loss via IHC predicts for subsequent cancer diagnosis and/or adverse pathologic outcomes in 40 cases of isolated intraductal lesions that did not meet current morphologic criteria for IDC-P (months 4-24).

1c. Validate PTEN IHC assays by correlating with *PTEN* fluorescence *in situ* hybridization (FISH) in 45 IDC-P lesions on tissue microarray (months 6-14).

Progress on Task 1: Expedited IRB approval for use of tissue specimens was received (11/15/13, "Development and Validation of Prognostic and Predictive Biomarkers in Human Prostate Tumor Specimens" JHU IRB # NA_00091198), however HRPO approval was not obtained until 5-22-15, which has delayed the timeline for progress somewhat. Because this precluded us from using CDMRP funds to being the project, in order to make progress, we used institutional funds to complete the preliminary work on Aim 1.

For Aim 1, tasks 1a and 1b have been completed and were recently published (See **Appendix**; Morais CL, Han JS, Gordetsky J, Nagar MS, Anderson AE, Lee S, Hicks JL, Zhou M, Magi-Galluzzi C, Shah RB, Epstein JI, De Marzo AM, Lotan TL. Utility of PTEN and ERG Immunostaining for Distinguishing High Grade PIN and Intraductal Carcinoma of the Prostate on Needle Biopsy. *American Journal of Surgical Pathology*, 2015; 39(2):169-78 PMC: PMC4293206.). In this study, we examined 50 prostate needle biopsies containing invasive tumor with intraductal carcinoma (**Figure 1**). Of these, 76% (38/50) showed PTEN loss and 58% (29/50) expressed ERG. Of biopsies containing *isolated* intraductal carcinoma, 61% (20/33) showed PTEN loss and 30% (10/33) expressed ERG. Of the borderline intraductal proliferations that did not qualify morphologic criteria as intraductal carcinoma, 52% (11/21) showed PTEN loss and 27% (4/15) expressed ERG. Of the borderline cases with PTEN loss, 64% (7/11) had carcinoma in a subsequent needle biopsy specimen, including 29% (2/7) with Gleason score 6 tumors, 29% (2/7) with a Gleason score 7 tumor, 14% (1/7) with a Gleason score 8 tumor and 29% (2/7) with definitive intraductal carcinoma. The remaining 36% (4/11) of cases with PTEN loss had either PIN or a repeat diagnosis of borderline lesion on subsequent biopsy. Of the PTEN intact cases, 50% (5/10) had a subsequent diagnosis of carcinoma, including 80% (4/5) with Gleason score 6 tumors, 20% (1/5) with Gleason score 7 cancer.

Thus, on needle biopsy, PTEN loss is common in morphologically identified intraductal carcinoma yet is very rare in high grade PIN. Borderline intraductal proliferations, especially those with PTEN loss, have a high rate of carcinoma, particularly higher grade (Gleason 7 or higher), on resampling. These results suggest that PTEN and ERG immunostaining may provide a useful ancillary assay to distinguish intraductal carcinoma from high grade PIN in needle biopsies, and we are currently using this assay in our clinical immunohistochemistry lab in this context.

For Task 1c, we have transferred the PTEN immunohistochemistry assay to a clinical platform which has eliminated all nuclear immunostaining in cases with negative cytoplasmic expression, suggesting that this finding was an artifact. To further validate the assay, we have studied PTEN immunohistochemistry versus PTEN fluorescence in situ hybridization (FISH) in an additional dataset, the Canary Retrospective Tissue Microarray Resource for prostate cancer specimens (n=731 radical prostatectomy specimens). In this overall cohort, PTEN IHC had high sensitivity and specificity for detection of PTEN gene deletions. The resulting manuscript has been reviewed and is under revision for *Modern Pathology* (Appendix 2). In this study, intact PTEN immunostaining was 91% specific for absence of *PTEN* gene deletion by FISH, (with 549/602 tumors with 2 copies of *PTEN* showing intact PTEN IHC) and 97% sensitive for homozygous *PTEN* deletion (with detectable PTEN protein loss in 65/67 homozygous tumors). PTEN IHC was 65% sensitive for detection of hemizygous *PTEN* deletion by FISH, with protein loss in 40/62 hemizygous tumors. IHC-guided FISH re-analysis in discordant cases, where IHC showed loss and FISH showed 2 intact copies of *PTEN*, revealed ambiguous IHC loss on re-review in 6% (3/53) cases and failure to analyze the same tumor area in 34% (18/53) cases. Of the remaining discrepant cases, 41% (13/32) revealed hemizygous (n=8) or homozygous (n=5) deletion that was focal in 94% (11/13) cases.

Because this study examines invasive tumors for correlation of FISH and IHC, we have determined the subset (~10%) of cases that include intraductal carcinoma in the sampled tumor spots on the TMA (**Figure 2**). These are currently being examined to ensure that the sensitivity and specificity of the IHC assay for detecting PTEN gene deletion is similar in intraductal carcinoma (the focus of task 1c) and invasive carcinoma. We are also examining a separate cohort of 14,000 prostatectomy specimens (the Martini Clinic cohort) where PTEN FISH (using 2-color probes) has already been reported (Krohn A, Diedler T, Burkhardt L, Mayer PS, De Silva C, Meyer-Kornblum M, et al. Genomic deletion of PTEN is associated with tumor progression and early PSA recurrence in ERG fusion-positive and fusion-negative prostate cancer. AJSP. 2012;181(2):401-12). We have performed PTEN IHC on the entirety of this cohort (30 TMAs) and are currently scoring it. In this cohort, approximately an additional 10% of tumor spots contain morphologically defined intraductal carcinoma. Thus we will separately examine the correlation between PTEN FISH and IHC in these spots in the analysis to accomplish the goals of task 1c.

Task 2: *Profile* the gene expression signature of IDC-P and compare it to that of HGPIN (months 8-36).

2a. Use laser capture microdissection (LCM) to obtain epithelial cells from morphologically-identified IDC-P and PIN occurring with concurrent Gleason 8 tumors and perform DASL and subsequent differential gene expression analysis to establish respective molecular signatures (months 12-36)

2b. Validate the top 3 promising candidate markers for distinguishing IDC-P from HGPIN at the RNA and protein levels using immunohistochemistry (IHC) and RNA *in situ* hybridization (ISH) on specimens collected in Task 1 (months 24-36).

Progress on Task 2: We awaited HRPO approval before using CDMRP funds to begin this work. Currently, we have identified ~50 cases of isolated PIN and IDC-P, each and have selected cases for sectioning in preparation for LCM. These cases have now been incorporated into a tissue microarray to facilitate validation of the top markers for Task 1. We are currently validating LCM technique and DNA isolation protocols to ensure robust nucleic acid recovery from these small samples, comparing two nucleic isolation methods (Qiagen AllPrep FFPE and QiAmp DNA) and two tumor enrichment methodologies (macrodissection and 0.6 mm tumor block cores). As seen in Table 1, these methods are fairly comparable, however RNA is obtained simultaneously from the AllPrep kit, thus we will proceed with this one. Task 2b will begin once we have completed task 2a.

Task 3: *Integrate* IDC-P into the molecular landscape of invasive carcinoma (months 18-36).

3a. Integrate the expression data for HGPIN and IDC-P into pre-existing, identically-collected datasets for high and low grade invasive tumors using Correspondence at the Top (CAT) plot analysis—supervised by Dr. Luigi Marchionni (months 18-28).

3b. Use the Ampliseq Comprehensive Cancer Panel to compare exomic sequences of 409 oncogenes/tumor suppressor genes in IDC-P with the sequences from the concurrent invasive cancer within each case (n=20 samples total) and confirm a subset of detected mutations using Taqman mutation detection assays (months 18-36).

3c. Use the Nanostring nCounter Cancer Copy Number Assay to compare copy number profile across 86 genes in IDC-P with those in concurrent invasive tumors (n=20 samples total) (months 18-36).

Progress on Task 3: Task 3a will begin after completion of Aim 2, above. Cases have been selected for Task 3b and a custom sequencing panel (Truseq) has been designed and is currently being tested on FFPE specimens. We have sequenced 80 invasive carcinoma cases with this panel to test whether it is robust and are currently curating the single nucleotide variant calls. Once we are satisfied with the platform, we will proceed to analyzing our relatively precious intraductal samples. For Task 3c, we have done some preliminary validation work with the Nanostring nCounter Cancer Copy Number Assay, but have been dissatisfied with the results as contamination by nearby stromal cells during microdissection appears to markedly influence the copy number calls. To mitigate this, we have validated another platform for copy number assessment that requires very low input DNA concentrations (80 ng), the Affymetrix Oncoscan platform (**Figure 3**). We have tested several cases with known genome wide copy number aberrations such as homozygous PTEN loss (via Affymetrix SNP 6.0 microarray) on this platform to assess the accuracy of the results with Oncoscan and the ease of use of their bioinformatics pipeline and we have been quite satisfied. Thus, once sample selection and DNA purification is complete, we will proceed with the copy number assessment in Task 3c.

4. KEY RESEARCH ACCOMPLISHMENTS:

- HRPO approval for this human tissue study has successfully be obtained (5-22-15).
- PTEN and ERG have been studied by immunohistochemistry as biomarkers of IDC-P and results have been published (Appendix 1: Morais CL, Han JS, Gordetsky J, Nagar MS, Anderson AE, Lee S, Hicks JL, Zhou M, Magi-Galluzzi C, Shah RB, Epstein JI, De Marzo AM, **Lotan TL**. Utility of PTEN and ERG Immunostaining for Distinguishing High Grade PIN and Intraductal Carcinoma of the Prostate on Needle Biopsy. *American Journal of Surgical Pathology*, 2015; 39(2):169-78 PMC: PMC4293206.
- The sensitivity and specificity of PTEN immunohistochemistry for PTEN gene deletion are high by comparison to PTEN FISH and the findings are the basis of a manuscript currently undergoing revision for *Modern Pathology*. (Appendix 2: Lotan TL, Wei W, Ludkovski O, Morais CL, Jamaspishvili T, Hawley ST et al. Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH. Under revision.)

5. **CONCLUSION:** PTEN and ERG can serve as useful immunohistochemical biomarkers of IDC-P and help to distinguish this aggressive lesion from indolent HGPIN in prostate biopsy specimens. PTEN immunohistochemistry has high sensitivity and specificity for detecting PTEN gene deletions in a large multi-institutional cohort of prostate cancer specimens. With HRPO approval in May, we are currently preparing to do expression analyses in order to select additional biomarkers and to do genomic analyses to integrate IDC-P into the molecular landscape invasive carcinoma.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

Morais CL, Han JS, Gordetsky J, Nagar MS, Anderson AE, Lee S, Hicks JL, Zhou M, Magi-Galluzzi C, Shah RB, Epstein JI, De Marzo AM, **Lotan TL**. Utility of PTEN and ERG Immunostaining for Distinguishing High Grade PIN and Intraductal Carcinoma of the Prostate on Needle Biopsy. *American Journal of Surgical Pathology*, 2015; 39(2):169-78 PMC: PMC4293206.

Lotan TL, Wei W, Ludkovski O, Morais CL, Jamaspishvili T, Hawley ST et al. Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH. Under revision for Modern Pathology. Presented in abstract form at USCAP 2015 meeting.

7. INVENTIONS, PATENTS AND LICENSES: None
8. REPORTABLE OUTCOMES: None
9. OTHER ACHIEVEMENTS:
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11. APPENDICES: See below

Utility of PTEN and ERG Immunostaining for Distinguishing High-grade PIN From Intraductal Carcinoma of the Prostate on Needle Biopsy

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Abstract: Intraductal carcinoma of the prostate and high-grade prostatic intraepithelial neoplasia (PIN) have markedly different implications for patient care but can be difficult to distinguish in needle biopsies. In radical prostatectomies, we demonstrated that PTEN and ERG immunostaining may be helpful to resolve this differential diagnosis. Here, we tested whether these markers are diagnostically useful in the needle biopsy setting. Separate or combined immunostains were applied to biopsies containing morphologically identified intraductal carcinoma, PIN, or borderline intraductal proliferations more concerning than PIN but falling short of morphologic criteria for intraductal carcinoma. Intraductal carcinoma occurring with concurrent invasive tumor showed the highest rate of PTEN loss, with 76% (38/50) lacking PTEN and 58% (29/50) expressing ERG. Of biopsies containing isolated intraductal carcinoma, 61% (20/33) showed PTEN loss and 30% (10/33) expressed ERG. Of the borderline intraductal proliferations, 52% (11/21) showed PTEN loss and 27% (4/15) expressed ERG. Of the borderline cases with PTEN loss, 64% (7/11) had carcinoma in a subsequent needle biopsy specimen, compared with 50% (5/10) of PTEN-intact cases. In contrast, none of the PIN cases showed PTEN loss or ERG expression (0/19). On needle biopsy, PTEN loss is common in morphologically identified intraductal carcinoma yet is very rare in high-grade PIN. Borderline intraductal proliferations, especially those with PTEN loss, have a high rate of carcinoma on resampling. If confirmed in larger prospective studies, these results suggest that PTEN and ERG immunostaining may provide a useful ancillary assay to distinguish intraductal carcinoma from high-grade PIN in this setting.

Key Words: prostatic intraepithelial neoplasia, intraductal carcinoma, prostatic carcinoma, PTEN, ERG

(*Am J Surg Pathol* 2015;39:169–178)

Intraductal carcinoma of the prostate and high-grade prostatic intraepithelial neoplasia (PIN) comprise the 2 main intraepithelial neoplastic lesions occurring in the prostate.[1] When diagnosed as isolated lesions on needle biopsies, these 2 entities have dramatically different implications for patient prognosis and care.[2–4] PIN is widely believed to be a nonobligate precursor lesion of invasive cancer, whereas intraductal carcinoma is a high-grade malignant lesion, likely representing retrograde intraductal/intra-acinar spread of high-grade invasive cancer in most cases.[2,4–11] Accordingly, PIN is frequently an isolated finding, occurring in biopsies without invasive carcinoma and, if not present in at least 2 to 3 separate biopsy cores, is not associated with an increased risk for cancer diagnosis on subsequent biopsies done within the following year.[3] In stark contrast, intraductal carcinoma is associated with underlying high-grade invasive carcinoma in $>90\%$ of cases.[2,11] Whereas many groups do not even recommend rebiopsy for isolated PIN occurring in a single needle core biopsy, most recommend definitive therapy (surgery or radiation) for intraductal carcinoma in a prostate needle core biopsy even without concurrent invasive carcinoma.[2,11] Further, in the presence of concurrent invasive carcinoma, accurate recognition of intraductal carcinoma is also critical as recent studies have established that the presence of this lesion is associated with adverse prognosis after surgery, radiation or neoadjuvant chemotherapy, or hormonal therapy.[12–16]

The distinction of PIN from intraductal carcinoma on needle core biopsy is currently based exclusively on morphologic assessment. Criteria for diagnosis of intraductal carcinoma (and distinction from PIN) have been

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proposed by several groups,[2,6,9] yet even with strict application of these criteria to needle biopsy specimens we and others have encountered a number of cases in which the intraepithelial proliferation shows borderline features, indeterminate between PIN and intraductal carcinoma.[10] Given the critical implications of the diagnosis for patient care, use of an ancillary molecular or immunohistochemical (IHC) test would be helpful in this setting. Recently, using radical prostatectomy specimens, we reported that PTEN protein loss occurs in the majority of morphologically identified intraductal carcinoma cases and was never observed in isolated high-grade PIN.[17] A similar study of borderline intraductal proliferations in radical prostatectomies showed that isolated lesions were entirely negative for ERG, whereas cancer-associated lesions or morphologically identified intraductal carcinoma were highly enriched (75%) for ERG expression.[18] Here, we examined whether immunostaining for PTEN, ERG, and basal cell markers (p63 and high–molecular weight keratin [HMWK]) would be useful to distinguish intraductal carcinoma from high-grade PIN in the more clinically relevant needle biopsy setting.

MATERIALS AND METHODS

Patient and Tissue Selection

This study, including tissue collection and IHC staining, was approved by the authors' Institutional Review Board. Prostate needle biopsy specimens containing intraductal carcinoma with concurrent invasive tumor (n = 50) were collected from the surgical pathology files of the Johns Hopkins Hospitals (JHH), the Cleveland Clinic, and Miraca Life Sciences. Needle biopsies containing isolated intraductal carcinoma (n = 33) without concurrent carcinoma were identified from the consultation files of JHH. All intraductal carcinoma cases were identified applying previously published morphologic criteria[2] and were defined as malignant epithelial cells filling large acini and prostatic ducts, with preservation of basal cells (confirmed by p63 and/or HMWK immunostaining) forming either (1) solid or dense cribriform patterns; or (2) loose cribriform or micropapillary patterns with either marked nuclear atypia (nuclear size >6 times normal or larger) or nonfocal comedonecrosis.

Borderline intraductal proliferations more concerning than high-grade PIN, but falling short of current criteria for intraductal carcinoma, were collected from the consultation files of JHH from 2010 to early 2012 (n = 60). Since 2010, we have diagnosed these cases descriptively as "atypical glands surrounded by basal cells where the differential diagnosis is between high-grade prostatic intraepithelial neoplasia (PIN) and intraductal carcinoma of the prostate" and recommended follow-up biopsies in all cases. The morphologic characteristics of these cases are described in the Results section below. None of these lesions were associated with concurrent infiltrating prostatic adenocarcinoma or a previous known diagnosis of such. Information regarding clinical follow-up was obtained from medical records or from correspondence with outside physicians.

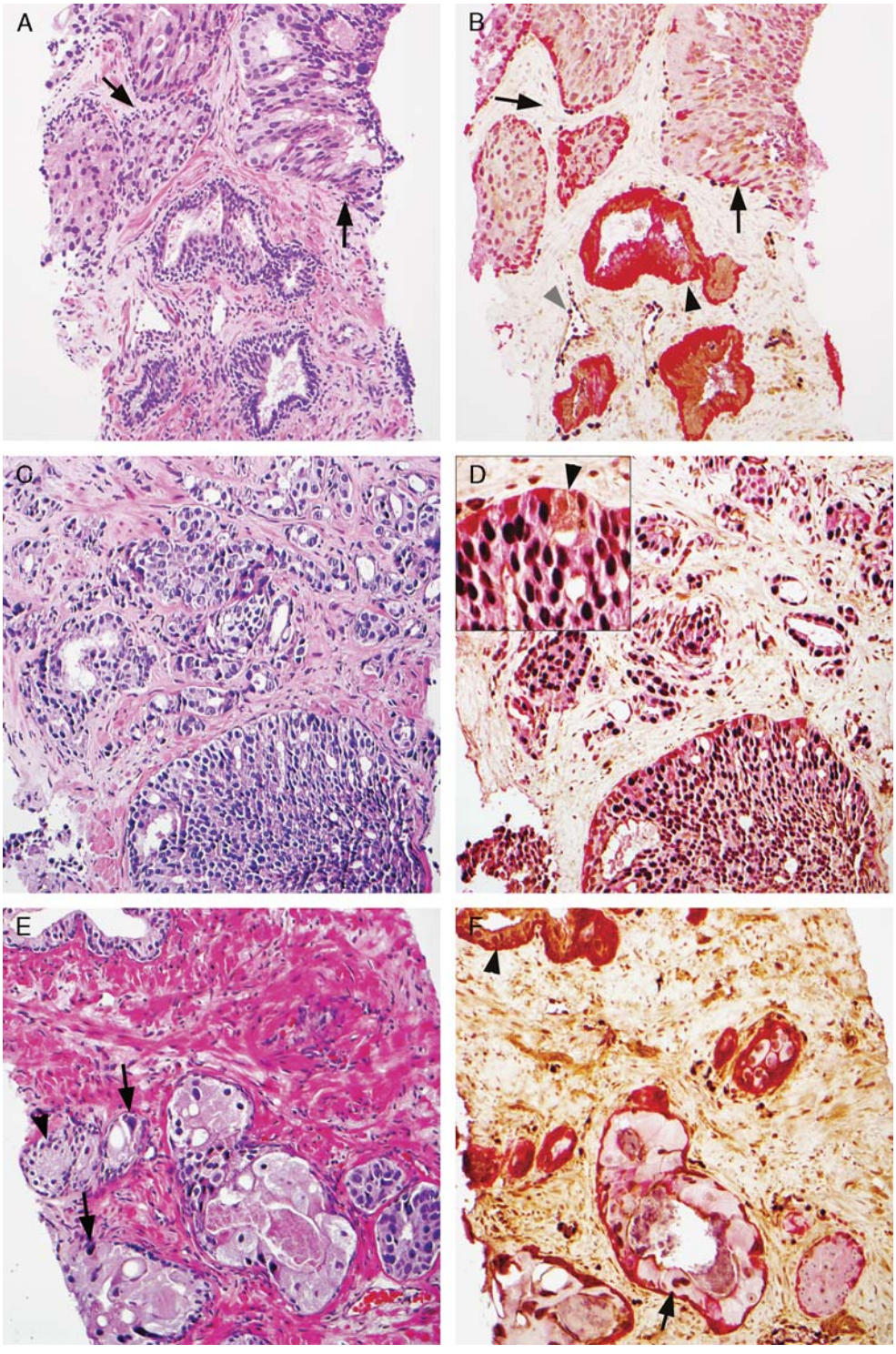
As a control group, we utilized needle biopsies containing high-grade PIN sampled either with (n = 7) or without (n = 12) concurrent carcinoma in additional cores. These cases were identified from the surgical pathology files of JHH from 2010 to 2012. High-grade PIN was defined as a tufted or micropapillary intraepithelial luminal proliferation, identifiable at low power, with nucleoli easily visualized at ×20 magnification.[1] No lesions with cribriform architecture were included in the high-grade PIN group for this study. Of the PIN cases occurring with concurrent carcinoma, 57% (4/7) occurred with Gleason score 3 + 3 = 6 carcinoma and 43% occurred with Gleason 3 + 4 = 7 carcinoma.

Immunohistochemistry

Immunostaining for PTEN, ERG, and basal cell markers was performed using 2 different strategies for cross-validation purposes. On the first subset of cases (30/50 cases of intraductal carcinoma with concurrent invasive adenocarcinoma, 10/33 cases of isolated intraductal carcinoma, and 13/21 cases of borderline intraductal proliferations), we used a 3-color chromogenic quadruple immunostain for PTEN, ERG, p63, and HMWK (34βE12 or CK903) that has been described previously.[17] In this assay, basal cells (p63 and HMWK) are labeled in red (alkaline phosphatase using Vector Red as chromogen), PTEN is labeled in brown (horseradish peroxidase using 3,3′-diaminobenzidine as chromogen), and ERG is labeled in purple (horseradish peroxidase using Vector VIP purple

FIGURE 1. PTEN loss and ERG expression are common in morphologically diagnosed intraductal carcinoma of the prostate on needle biopsy. A, Dense cribriform to solid architecture in an isolated intraductal carcinoma case (arrows). B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates PTEN loss in intraductal carcinoma (arrows) compared with nearby benign gland (black arrowhead). ERG is expressed in nuclei of intraductal proliferation, although it is less intense than nearby endothelial cells (gray arrowhead). C, Dense cribriform intraductal carcinoma with nearby invasive carcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates PTEN loss and ERG expression in intraductal carcinoma cells (inset) relative to entrapped benign cells (inset, arrowhead). The surrounding invasive carcinoma is concordant with the intraductal carcinoma for these markers. E, Intraductal carcinoma with marked cytologic atypia. Although this case does not show dense cribriform or solid intraductal proliferation, it qualifies as intraductal carcinoma because of the presence of atypical nuclei (arrows) >6× the size of surrounding benign nuclei (arrowhead). F, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (E) demonstrates PTEN loss in intraductal carcinoma cells (arrow) relative to nearby benign glands (arrowhead). ERG is also expressed in this case.

as chromogen). To further validate the quadruple immunostain (and in part because the p63 antibody clone 4A4 used in the quadruple immunostain became commercially unavailable during the course of the study), we performed the PTEN, ERG, and HMWK immunostaining analyses individually on adjacent tissue levels on the remainder of the cases, using the same antibody clones as in the quadruple stain, in addition to the previously validated staining protocols.[19,20] Rates of PTEN/ERG staining were nearly identical for each class of lesions using the 2 immunostaining



strategies, further validating the quadruple immunostain's equivalency to the individual stains.

Interpretation of IHC

Cytoplasmic PTEN and nuclear ERG protein were visually scored using a previously validated dichotomous scoring system[19] by a urologic pathologist (T.L.L.). All lesional glands were scored that met morphologic criteria for intraductal carcinoma, borderline intraductal proliferation, or high-grade PIN, based on side-by-side comparisons with a hematoxylin and eosin–stained section. Lesions were scored only if the presence of basal cells could be documented by p63 and/or 34βE12 staining. As previously described,[17,19] cytoplasmic staining for PTEN was classified as negative if the intensity was markedly decreased or entirely negative across >90% of lesional epithelial cells within each gland when compared with the surrounding benign glands and/or stroma, which provide an internal positive control. In a previous study, we found that using this scoring system, PTEN IHC was 100% sensitive and 97.8% specific for *PTEN* genomic loss across a panel of 58 cell lines and between 75% and 86% sensitive for *PTEN* genomic loss in 119 genetically characterized prostate tumor tissues.[19]

Staining for nuclear ERG was assessed in comparison with stromal endothelial cell staining, which provided an internal positive control for ERG in each section. Similarly, adjacent benign glands provided an internal negative control for ERG staining in all cases. Using cutoffs found to be nearly 90% specific for *ERG* gene rearrangement in a prior study,[20] staining for ERG was considered positive if any lesional cells showed nuclear positivity, even those with somewhat weaker staining when compared with surrounding endothelial cells, and negative if no lesional cells were positive.

Statistical Analysis

Fisher exact tests were used to determine the correlation of PTEN and ERG protein expression with one another.

RESULTS

PTEN and ERG Expression in Intraductal Carcinoma and High-grade PIN

Intraductal carcinoma occurring with concurrent invasive tumor showed the highest rate of PTEN protein loss, with 76% (38/50) of cases lacking PTEN protein (Fig. 1, Table 1). In total, 58% (29/50) of these cases expressed ERG. ERG expression was seen in 66% (25/38) of the PTEN loss cases, compared with only 33% (4/12) of the PTEN-intact cases ($P = 0.091$ by the Fisher exact test; Table 2). Overall, 70% (35/50) of cases had concurrent invasive carcinoma present on the same needle core as the intraductal tumor available for analysis. Of these cases, 97% (34/35) showed concordant PTEN and ERG staining between the intraductal and invasive carcinoma. The one discordant case showed PTEN loss in the intraductal component with intact PTEN in the invasive component in the background of negative ERG staining in both components.

TABLE 1. Rate of PTEN Loss and ERG Expression in a Spectrum of Intraepithelial Prostate Proliferations

Intraepithelial Lesion	PTEN Loss (n [%])	ERG Expression (n [%])
Intraductal carcinoma with concurrently sampled invasive carcinoma	38/50 (76)	29/50 (58)
Isolated intraductal carcinoma	20/33 (61)	10/33 (30)
Borderline intraductal proliferations	11/21 (52)	4/15 (27)
PIN with concurrently sampled invasive carcinoma	0/7 (0)	0/7 (0)
Isolated PIN	0/12 (0)	0/12 (0)

Of the needle biopsies containing isolated intraductal carcinoma, 61% (20/33) showed PTEN protein loss and 30% (10/33) expressed ERG. Of the cases with PTEN loss, 50% (10/20) expressed ERG protein, whereas none of the PTEN-intact cases expressed ERG (0/13, $P = 0.0022$ by the Fisher exact test; Fig. 1, Table 3). In contrast, of the high-grade PIN cases occurring with concurrent carcinoma in additional cores, 0% (0/7) showed PTEN loss or ERG expression. Similarly, of the isolated high-grade PIN cases, 0% (0/12) showed PTEN loss or ERG protein expression (Fig. 2, Table 1).

Clinical-pathologic Features of Borderline Intraductal Proliferations Falling Short of Intraductal Carcinoma

We identified 60 cases of borderline intraductal proliferations falling short of current criteria for intraductal carcinoma in our urologic consultation case files from 2010 to early 2012. We limited our search to this period because 2010 was when we first began to formally diagnose these lesions, and we wanted old enough cases to have at least 2 years of clinical follow-up. These cases were characterized by: (1) lumen-spanning proliferation with loose cribriform architecture beyond what would normally be seen in high-grade PIN but lacking significant nuclear pleomorphism or necrosis to qualify for IDC-P (these cases are morphologically similar to those described in our previous radical prostatectomy study as "intraductal cribriform proliferations,"[17]) (Fig. 3A); and/or (2) atypical nuclei with significant pleomorphism but falling short of what is required for a diagnosis of IDC-P (< 6 times larger than adjacent normal epithelial cells) (Fig. 3C); and/or (3) dense cribriform or solid proliferation of atypical cells in incompletely represented large ducts on the edge of core biopsy specimens (Fig. 3E). The majority of cases showed >1 of these features.

Of the 60 cases of borderline lesions, information about subsequent tissue sampling was available in 60% (36/60). Thirty-five of these patients underwent rebiopsy, and 1 underwent an immediate radical prostatectomy despite the fact that he lacked a tissue diagnosis of carcinoma. The remainder of the patients (40%) failed to follow-up with their original urologist or elected to forgo an additional follow-up biopsy despite our recommendation.

TABLE 2. PTEN and ERG Status of Intraductal Carcinoma Sampled With Invasive Carcinoma on Needle Biopsy ($P = 0.091$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	8	4
PTEN loss	13	25

TABLE 3. PTEN and ERG Status of Isolated Intraductal Carcinoma on Needle Biopsy ($P = 0.0022$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	13	0
PTEN loss	10	10

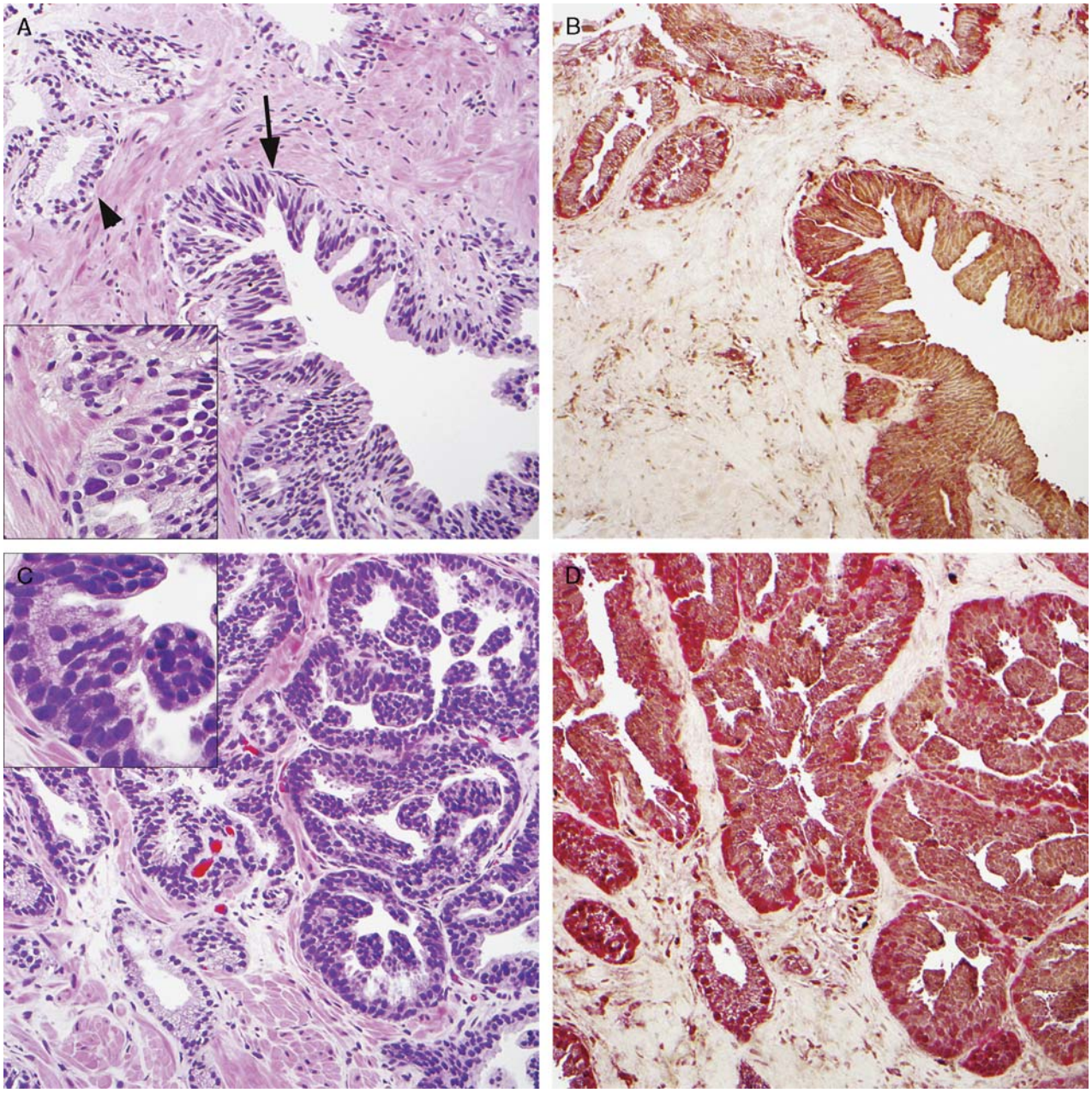


FIGURE 2. PTEN loss and ERG expression are not seen in morphologically diagnosed high-grade PIN on needle biopsy. A, High-grade PIN with tufted architecture (arrow). Nuclear enlargement and nucleoli are apparent at low magnification (arrow) compared with surrounding benign glands (arrowhead). Nucleoli are easily visible (inset). B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates intact PTEN and absence of ERG staining. C, High-grade PIN with micropapillary architecture. This case contained concurrent invasive adenocarcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates intact PTEN and absence of ERG staining. Nucleoli are easily visible (inset).

Of the 36 patients with additional tissue sampling after a diagnosis of this borderline lesion, the median age was 70 years (range: 56 to 85 y). The number of cores involved by the borderline intraductal proliferation in each case ranged from 1 to 5, with a median of 1. There was a separate focus of atypical glands, suspicious for carcinoma, in 7 of 36 cases (19%). No concurrent invasive carcinoma was diagnosed in any case. The median interval to rebiopsy overall was 4 months, ranging between 0.6 and 3 years. On rebiopsy (or in 1 case, subsequent radical prostatectomy), 50% (18/36) of patients were diagnosed with prostatic carcinoma, with 83% (15/18) showing invasive tumor and 17% (3/18) showing definitive IDC-P. For these patients with a subsequent diagnosis of carcinoma, the median interval to rebiopsy was 5.6 months. For patients with invasive tumor on rebiopsy, 53% (8/15) had a Gleason score of 6, 33% (5/15) had a Gleason score of 7, and 13% (2/15) had a Gleason score of 8. For 2 of the patients with Gleason score 6 carcinoma, the tumor was diagnosed on a second follow-up biopsy, following a rediagnosis of borderline intraductal lesion on the first follow-up biopsy. These were the only 2 patients who had undergone 2 rebiopsies at the time of follow-up. Of the remaining patients, 44% (8/18) showed a borderline intraductal proliferation once again on rebiopsy, 17% (3/18) had a diagnosis of atypical glands, suspicious for prostatic carcinoma, 28% (5/18) showed high-grade PIN on rebiopsy, and 11% (2/18) had a benign diagnosis on rebiopsy.

PTEN and ERG Expression in Borderline Intraductal Proliferations Falling Short of Intraductal Carcinoma

Of the 36 patients who underwent additional tissue sampling after the diagnosis of a borderline lesion, tissue was available for PTEN immunostaining in 58% (21/36) of cases and ERG immunostaining in 42% (15/36). Of these cases, 52% (11/21) showed PTEN protein loss and 27% (4/15) expressed ERG protein (Fig. 3, Table 1). Fifty percent (4/8) of the cases showing PTEN loss expressed ERG compared with 0% (0/7) of the PTEN protein intact cases (Tables 4, $P = 0.0769$ by the Fisher exact test). Of the cases with PTEN loss, 64% (7/11) had carcinoma sampled on a subsequent biopsy, including 29% (2/7) with a Gleason score 6 tumor, 29% (2/7) with a Gleason score 7 tumor, 14% (1/7) with a Gleason score 8 tumor, and 29% (2/7) with definitive intraductal carcinoma. The remaining 36% (4/11) of cases with PTEN loss had either PIN or a repeat diagnosis of borderline lesion on subsequent biopsy. Of the PTEN-intact cases, 50% (5/10) had a subsequent diagnosis of carcinoma, including 80% (4/5) with a Gleason score 6 tumor and 20% (1/5) with Gleason score 7 cancer.

DISCUSSION

Currently, the diagnosis of intraductal carcinoma remains a morphologic one, thus sensitive and specific criteria to accurately distinguish this lesion from common high-grade PIN are essential. As originally defined by McNeal and Yemoto,[6] intraductal carcinoma was characterized by ducts or acini lined by basal cells with an epithelial layer showing cytologic features of moderate-grade to high-grade dysplasia with the additional requirement that luminal extensions of the epithelial lining completely bridged the luminal diameter either as trabeculae or cell masses. Cohen et al[9] proposed 5 major and several minor criteria that built on the original McNeal classification. In contrast to McNeal's criteria, Cohen's criteria included the expansile nature of the lesion, with involved glands more than twice the diameter of normal surrounding peripheral zone glands. Minor criteria included glands that branch at right angles, have smooth contours, and included a dual cell population with more atypical cells at the periphery and maturation toward the center of the lumen. Subsequent criteria put forth by the Epstein group (and utilized in this study) were the most stringent, requiring that the intraluminal proliferation either show a dense cribriform or solid architecture or, if not, have marked cytologic atypia defined on the basis of nuclear size or have comedonecrosis.[2] Application of these criteria to define isolated intraductal carcinoma in prostate needle biopsies was >90% sensitive for detection of underlying invasive carcinoma in subsequent radical prostatectomy specimens.[11]

Given the vastly different clinical implications of the diagnosis of intraductal carcinoma versus the diagnosis of PIN, most morphologic criteria for intraductal carcinoma have emphasized specificity over sensitivity. High-grade PIN most commonly has a tufted or micropapillary architecture with moderate, but not marked, cytologic atypia and nucleoli easily visible at ×20 magnification.[1] In contrast to intraductal carcinoma, solid architecture and comedonecrosis are never seen in PIN; however, the clas-

FIGURE 3. PTEN and ERG expression in borderline intraepithelial proliferations more concerning the PIN, but insufficient for a diagnosis of intraductal carcinoma using current morphologic criteria. A, Borderline proliferation with loose cribriform architecture, unusual for PIN, but insufficient for diagnosis of intraductal carcinoma. B, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (A) demonstrates PTEN loss relative to adjacent benign cells (inset shows involved gland from a different area of the core; arrowhead demonstrates a nearby benign gland) and diffuse expression of ERG. C, Borderline proliferation with substantial cytologic atypia (arrow) but lacking sufficient atypia to qualify as intraductal carcinoma. D, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (C) demonstrates pagetoid spread of PTEN-negative, ERG-positive cells (arrow). E, Borderline proliferation with dense cribriform architecture, which is highly suspicious for intraductal carcinoma but insufficiently represented at the edge of the needle core. F, Quadruple immunostain for PTEN (brown), ERG (purple), and basal cells (red) on case in (E) demonstrates retention of PTEN and lack of ERG expression in the proliferation.

sification of more loosely cribriform or lumen-spanning intraductal proliferations has been controversial.[2,4,6,7,9–11] Should all lumen-spanning intraductal lesions be considered intraductal carcinoma? Does cribriform PIN exist? Previous studies of so-called "atypical cribriform lesions" using radical prostatectomy specimens have found that the vast majority occur within close proximity to invasive, frequently high-grade, carcinoma.[10] Although a minority occur in isolation from invasive carcinoma and fail to satisfy the criteria for IDC-P, these lesions are relatively rare. Interestingly, although ERG was rearranged in approximately three quarters of atypical cribriform lesions occurring in close proximity to invasive tumors, ERG rearrangement was not seen in isolated atypical cribriform

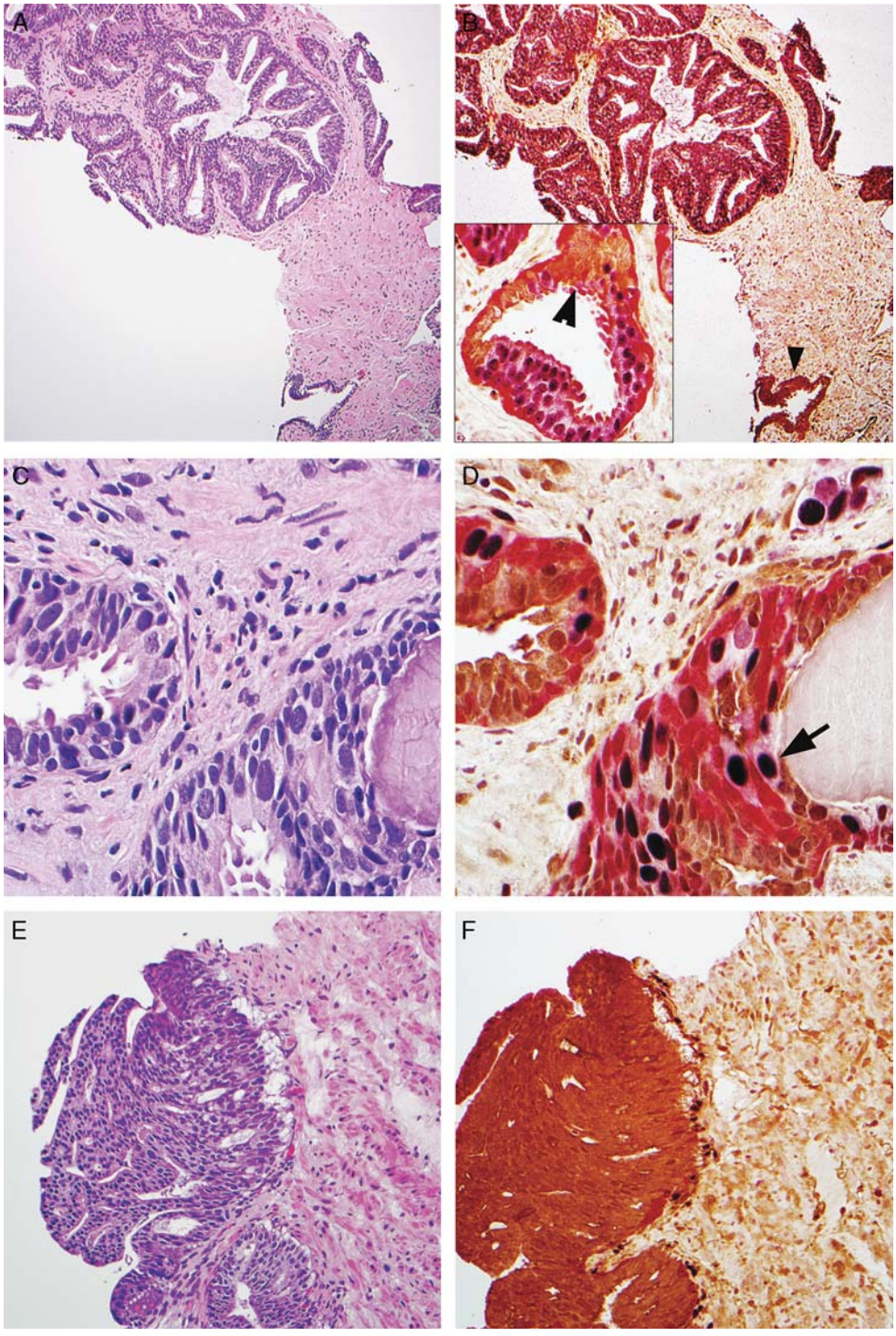


TABLE 4. PTEN and ERG Status of Borderline Intraductal Proliferations Falling Short of Morphologic Diagnosis of Intraductal Carcinoma ($P = 0.0769$ by Fisher Exact Test)

	ERG Negative	ERG Positive
PTEN intact	7	0
PTEN loss	4	4

lesions, suggesting that they may be molecularly distinct and most similar to PIN.[18] Thus, the authors concluded that, although rare true cases of cribriform PIN may exist, these cases are quite uncommon, and, when sampled on needle biopsy, all cribriform intraductal proliferations falling short of intraductal carcinoma should at least undergo a rebiopsy to exclude unsampled carcinoma.

These data and others on radical prostatectomy specimens strongly suggested that ancillary molecular tests may have significant utility for resolving the differential diagnosis of these difficult cribriform lesions.[18,21,22] Because ERG fluorescence in situ hybridization is expensive and time-consuming to perform, and ERG rearrangement can be seen in a subset of conventional PIN cases, in previous work, we focused on the utility of combined PTEN and ERG IHC to distinguish PIN from intraductal carcinoma in radical prostatectomy specimens.[17] In our previous study, we showed that PTEN loss by IHC (which is highly concordant with the presence of an underlying *PTEN* deletion) occurs in >80% of intraductal carcinoma (defined by Epstein criteria) and was not seen in morphologically typical high-grade PIN lesions from grade-matched and stage-matched specimens. The common occurrence of PTEN loss in intraductal carcinoma not only provides a potential marker for this lesion but also suggests a molecular mechanism for the aggressive behavior of tumors associated with intraductal carcinoma. Interestingly, in our previous study, we also examined loose, lumen-spanning cribriform intraepithelial proliferations that fell short of intraductal carcinoma criteria but were adjacent to invasive carcinoma. All of these lesions had loss of PTEN, strongly suggesting that we may be underrecognizing some cases of intraductal carcinoma using current criteria. In this study, ERG was positive in a subset of intraductal carcinomas and frequently concordant with PTEN loss.

Because all previous molecular studies of intraductal lesions have been performed in radical prostatectomy specimens, in which the distinction between intraductal carcinoma and PIN is often straightforward, in the current study we examined these markers in the more clinically relevant setting of prostate needle biopsies. In line with our results from the radical prostatectomy study, we found that over three quarters of morphologically identified intraductal carcinomas occurring with concurrent invasive adenocarcinoma show PTEN protein loss. In isolated intraductal carcinoma sampled without concurrent invasive tumor on needle biopsy, the rate of PTEN loss is similar at 60%. It should be noted that in our previous studies using this same method of PTEN detection by IHC, frequencies of PTEN loss approaching 75% were not seen even in high-grade primary and hormone-naive metastatic prostate carcinomas.[19,23–25] This provides further support for the somewhat unique biological nature of intraductal carcinoma and associated invasive lesions. Strikingly, PIN sampled on needle biopsy with or without concurrent invasive carcinoma did not show PTEN loss in the present study, a finding consistent with our earlier study of PIN in radical prostatectomy specimens.[17] ERG was expressed in 47% (39/83) of intraductal carcinomas overall on biopsy, and its expression was more commonly seen in cases with PTEN loss (60% or 35/58) than those without PTEN loss (16% or 4/25; $P = 0.0002$ by the Fisher exact test), as has been previously reported.[26–29] Interestingly, we did not see ERG expression in the 19 cases of isolated PIN examined in this biopsy study. Prior studies have shown ERG expression in up to 20% of PIN cases; however, it is more commonly seen in PIN adjacent to invasive cancer[30,31] or in isolated PIN diagnosed on needle biopsies from patients with a subsequent diagnosis of invasive cancer.[32]

Taken together, our data suggest that PTEN IHC, either alone or in combination with ERG, may be useful as an ancillary test to distinguish intraductal carcinoma from PIN on prostate needle biopsy. To begin to formally test this hypothesis, we also studied the outcomes of difficult-to-classify borderline intraductal lesions sampled without concurrent carcinoma on needle biopsy. In this category, we included the controversial loose cribriform lesions described above, in addition to intraepithelial lesions with substantial cytologic atypia (but insufficient for a diagnosis of intraductal carcinoma) and lesions only partially represented at the edge of a biopsy core. As a group, these borderline intraductal lesions are analogous to those classified as atypical glands suspicious for carcinoma (ATYP) or atypical small acinar proliferations (ASAP) in that they do not appear to represent an entity in and of themselves, yet their presence in a needle biopsy signifies an increased risk for carcinoma on subsequent biopsies.[1,3,33] In the current series, the risk for carcinoma diagnosis on subsequent biopsy was 50%, slightly higher than that seen after the diagnosis of atypical glands suspicious for carcinoma.[3] Importantly, however, and in contrast to atypical glands suspicious for carcinoma, almost half of borderline intraductal cases with a subsequent diagnosis of invasive carcinoma showed Gleason score of 7 or higher, suggesting that many of these tumors are clinically significant and that a prompt diagnosis is required.

These data strongly suggest that current morphologic criteria for intraductal carcinoma on prostate needle biopsy, although quite specific, may not be optimally sensitive. Thus, we took the first steps to retrospectively examine the utility of PTEN and ERG IHC to predict outcomes in these borderline intraductal lesions. We found that borderline lesions with PTEN loss on needle biopsy had a 64% risk for definitive carcinoma (intraductal or invasive) on subsequent biopsy, a slightly higher risk than seen in the overall population of borderline lesions, and a rate substantially higher than that seen after a diagnosis of a small focus of atypical glands suspicious for carcinoma (ATYP or ASAP).[1,3,33] Although this rate

of carcinoma on rebiopsy was somewhat higher than that seen in the PTEN-intact lesions (64% vs. 50%) or for borderline lesions overall (50%), the current study of borderline lesions has a number of limitations that suggest it is not yet ready for routine clinical use in this context. Perhaps most importantly, it is limited by its modest sample size, as clinical follow-up with available additional tissue for immunostaining was difficult to obtain in our consultation-enriched study population. In addition, our study is limited by the fact that, because of current standards of care, all of the patients were followed up with a needle biopsy, which has limited sensitivity for detection of cancer compared with more thorough examination of a radical prostatectomy specimen. Thus, even if 100% of patients with borderline lesions showing PTEN loss had underlying carcinoma, we would not expect to detect all of these in a single follow-up needle biopsy. The increasing use of magnetic resonance imaging–guided biopsy is rapidly improving the pervasive issue of tumor-undersampling with transrectal ultrasound biopsies. Thus, it is our hope that larger future studies may improve upon our current data and are certainly required before PTEN loss may be used (in combination with morphologic evaluation) to recommend definitive therapy in a borderline intraductal lesion.

Despite these limitations, this study represents the first to use validated molecular markers as an ancillary test to help classify difficult intraepithelial lesions in the prostate with clinical follow-up. Given the clinical significance of distinguishing intraductal carcinoma from high-grade PIN, ancillary molecular tests to help resolve ambiguous cases would be quite valuable to the practicing pathologist. Although these stains (as with all IHC) must always be interpreted in the context of morphology, they may be especially helpful adjunct markers for pathologists who do not see large volumes of urologic material and are less comfortable with the diagnosis of intraductal carcinoma on morphologic grounds alone. Importantly, PTEN loss is only seen in 60% to 70% of classic intraductal carcinoma lesions using current morphologic criteria. This means that if PTEN is intact, this does not rule out a diagnosis of intraductal carcinoma, reducing the negative predictive value of the test, and reinforcing the requirement for morphologic evaluation. In contrast, if PTEN is lost, the positive predictive value is reasonably high, as PTEN loss is rarely if ever seen in morphologically identified PIN.

Further, PTEN loss in an intraepithelial lesion would not only potentially help distinguish it from PIN but, even in a morphologically identifiable case of intraductal carcinoma, would strongly suggest the presence of a concurrent underlying invasive carcinoma with PTEN loss, as these lesions are highly concordant for PTEN status.[17] As we have previously shown that PTEN loss in invasive tumors is strongly associated with higher stage and grade,[19,25] worse outcomes,[19,23,25] and upgrading,[34] this is potentially valuable information to have on a needle biopsy. Although admittedly a small sample size, these data are supported by the current study. More than 70% (5/7) of the patients with borderline intraductal proliferations showing PTEN loss and a subsequent diagnosis of carcinoma had Gleason 7 or higher tumors or intraductal carcinoma (almost invariably associated with Gleason 7 or higher invasive carcinoma[2,11]). In contrast, only 20% (1/5) of the borderline intraductal proliferations with intact PTEN and subsequent carcinoma were diagnosed with Gleason 7 tumor and none with definitive intraductal carcinoma. Given that PTEN loss is only about 60% to 70% sensitive for the detection of intraductal carcinoma on needle biopsy, borderline lesions with intact PTEN not meeting current morphologic criteria for intraductal carcinoma would still need to be followed up with additional biopsies. However, if supported by larger prospective studies, these preliminary results suggest that this simple IHC assay for PTEN may ultimately be useful to help select cases that would benefit from immediate definitive therapy.

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Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH

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Key Words: Prostatic carcinoma; PTEN, fluorescence in situ hybridization, immunohistochemistry, radical prostatectomy, biomarker

Running Title: Analytic Validation of PTEN IHC

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Abstract:

PTEN loss is a promising prognostic and predictive biomarker in prostate cancer. Because it occurs most commonly via *PTEN* gene deletion, we developed a clinical-grade, automated and inexpensive immunohistochemical (IHC) assay to detect PTEN loss. We studied the sensitivity and specificity of PTEN IHC relative to 4-color fluorescence *in situ* hybridization (FISH) for detection of *PTEN* gene deletion in a multi-institutional cohort of 731 primary prostate tumors. Intact PTEN immunostaining was 91% specific for absence of *PTEN* gene deletion, (549/602 tumors with 2 copies of the *PTEN* gene by FISH showed intact expression of PTEN by IHC) and 97% sensitive for presence of homozygous *PTEN* gene deletion (absent PTEN protein expression by IHC in 65/67 tumors with homozygous deletion). PTEN IHC was 65% sensitive for presence of hemizygous *PTEN* gene deletion, with protein loss in 40/62 hemizygous tumors. We reviewed the 53 cases where IHC showed PTEN protein loss and FISH showed 2 intact copies of the *PTEN* gene. On re-review, there was ambiguous IHC loss in 6% (3/53) and failure to analyze the same tumor area by both methods in 34% (18/53). Of the remaining discordant cases, 41% (13/32) revealed hemizygous (n=8) or homozygous (n=5) *PTEN* gene deletion that was focal in most cases (11/13). The remaining 19 cases had 2 copies of the *PTEN* gene by FISH, representing truly discordant cases. Our automated PTEN IHC assay is a sensitive method for detection of homozygous *PTEN* gene deletions. IHC screening is particularly useful to identify cases with heterogeneous *PTEN* gene deletion in a subset of tumor glands. Mutations, small insertions or deletions and/or epigenetic or microRNA-mediated mechanisms may lead to PTEN protein loss in tumors with normal or hemizygous *PTEN* gene copy number.

Introduction:

PTEN is the most commonly lost tumor suppressor gene in prostate cancer (1-5) and is a promising prognostic biomarker for poor clinical outcomes (6-17). Since the *PTEN* gene is almost always lost by genomic deletion of the entire gene in prostate tumors, fluorescence *in situ* hybridization (FISH) has traditionally been the gold standard assay to detect *in situ PTEN* loss in tumor tissue. However the relatively recent availability of reliable rabbit monoclonal antibodies for detection of PTEN protein has enabled the development of highly validated immunohistochemistry (IHC) protocols to detect PTEN loss in prostate cancer (9, 18). IHC-based detection of PTEN loss in prostate cancer is less expensive and less time-consuming than FISH for the routine screening of prostate tumor specimens, making it easier to adapt to the current pathology work flow for risk assessment in prostate cancer. In addition, since *PTEN* loss is commonly subclonal and heterogeneous in primary prostate tumors (9, 19-21), detection of *PTEN* gene deletion by FISH can be technically challenging in some cases and screening for focal loss may be more easily accomplished by IHC. Finally, there is emerging evidence that in addition to genetic deletion, PTEN protein levels may be compromised by mutations in the gene or microRNA- or epigenetic-regulated mechanisms which would not be detectable by FISH (9, 22-24).

We previously optimized and validated a PTEN IHC assay for the detection of PTEN loss in prostate cancer specimens (9), and PTEN loss by this assay correlated with increased risk of biochemical recurrence in a case-control cohort of patients undergoing radical prostatectomy (12) and with risk of progression and metastasis in two independent high risk surgical cohorts (9, 11). Though originally performed

manually, we have recently transferred this assay to a clinical-grade automated immunostaining platform that may be run in any CLIA-certified pathology laboratory. Using this assay, we recently reported that PTEN loss is associated with reduced recurrence free survival in a multi-institutional cohort of surgically treated patients (25) with low inter-observer variability (κ>0.900). *PTEN* gene deletion by FISH has also been recently reported in a subset of this same cohort and correlated with recurrence free survival (17). Here, to analytically validate our clinical-grade PTEN IHC assay, we compared the performance of the automated IHC assay to *PTEN* FISH in this cohort, one of the largest multi-institutional cohorts to be studied by both techniques. We demonstrate that our IHC assay shows robust sensitivity and specificity for detection of homozygous *PTEN* gene deletion.

Methods:

Subject selection and tissue microarray design: The Canary Foundation Retrospective Prostate Tissue Microarray Resource has been described in detail elsewhere (26). Briefly, it is a multicenter, retrospective prostate cancer tissue microarray (TMA) created as a collaborative effort with radical prostatectomy (RP) tissue from six academic medical centers: Stanford University, University of California San Francisco, University of British Columbia, University of Washington (including tissues from University of Washington and a separate cohort from the Fred Hutchinson Cancer Research Center), University of Texas Health Science Center at San Antonio, and Eastern Virginia Medical School. Tumor tissue from 1275 patients was selected for the TMA using a quota sampling plan, from RP specimens collected between 1995 and 2004. A starting date of 1995 was selected to enrich for cases occurring after the implementation of PSA screening. There was no central pathology review in this cohort. The TMA included samples from men with (a) recurrent prostate cancer; (b) nonrecurrent prostate cancer; and (c) unknown outcome due to inadequate follow-up time (ie, censoring). Recurrent cases of Gleason score 3+3=6 and 3+4=7 were relatively over-sampled as well as non-recurrent cases with Gleason score 4+4=8, in order to improve power to detect biomarkers providing prognostic information independent of Gleason score.

Each site built 5 TMAs, each containing tumor tissue from 42 patients (210 patients from each contributing site). Each tumor was sampled in triplicate, utilizing 1 mm cores and an additional core of histologically benign peripheral zone tissue was

included for each patient as a control. Recurrent and non-recurrent patients were distributed randomly across all TMAs.

Immunohistochemistry assays**:** PTEN immunohistochemistry (IHC) was performed on the CFRPTMR cohort as recently reported (25)**.** Briefly, the protocol uses the Ventana automated staining platform (Ventana Discovery Ultra, Ventana Medical Systems, Tucson, AZ) and a rabbit anti-human PTEN antibody (Clone D4.3 XP; Cell Signaling Technologies, Danvers, MA). We previously validated a manual version of this assay using the same antibody in genetically characterized cell lines and prostate tumor tissue, showing strong correlation of the IHC with *PTEN* gene copy number by 2-color FISH and high resolution SNP array analysis (9) and good correlation with 4-color FISH in a small cohort of needle biopsy specimens (27). To prove equivalence between the manual and automated assays, we also examined a test TMA containing 50 prostate cancer cases with known PTEN protein status (including more than 30 with PTEN protein loss) by manual staining and found 100% concordance between the PTEN protein status on the manual and automated platforms.

Immunohistochemistry scoring: After staining for PTEN, all TMAs were scanned at 20x magnification (Aperio, Leica Microsystems, Buffalo Grove, IL) and segmented into TMAJ for scoring (http://tmaj.pathology.jhmi.edu/). PTEN protein status was blindly and independently scored by two trained pathologists (TLL and CLM) using a previously validated scoring system (see below). Overall, there was “very good” agreement between independent reviewers, with 97% agreement over 2784 cores scored by both reviewers (25).

A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly decreased or entirely negative across >10% of tumor cells compared to surrounding benign glands and/or stroma, which provide internal positive controls for PTEN protein expression (9). If the tumor core showed PTEN protein expressed in >90% of sampled tumor glands, the tumor was scored as PTEN intact. If PTEN was lost in <100% of the tumor cells sampled in a given core, the core was annotated as showing heterogeneous PTEN loss in some, but not all, cancer glands (focal loss). Alternatively, if the core showed PTEN loss in 100% of sampled tumor glands, the core was annotated as showing homogeneous PTEN loss. Finally, a small percentage of cores were scored as having ambiguous PTEN IHC results. This occurred when the intensity of the tumor cell staining was light or absent in the absence of evaluable internal benign glands or stromal staining. The percent of tissue cores with ambiguous scoring for PTEN IHC was fairly constant across 6 of the 7 institutions included in the Canary TMA cohort and varied from 0.7%-5.3% (25).

For statistical analysis, each patient's tumor sample was scored for the presence or absence of PTEN loss by summarizing the scores of each individual sampled core from that tumor. A patient's tumor was designated as having heterogeneous PTEN loss if at least one tumor core showed heterogeneous PTEN loss, or alternatively, if at least one core showed heterogeneous or homogeneous PTEN loss and at least one core showed PTEN intact in tumor cells. A patient's tumor was scored as showing homogeneous PTEN loss if all sampled tumor cores showed homogeneous PTEN loss. Finally, a patient's tumor was scored as having PTEN intact if all sample tumor cores showed intact PTEN in sampled tumor glands.

Initial blinded analysis of *PTEN* FISH: PTEN FISH was performed as previously described for a subset of this cohort (17). Briefly, the PTEN Del TECT FISH utilizes a four color probe combination as described. Probes were supplied by CymoGenDx LLC (New Windsor, NY) as follows: centromeric copy control probe - CYMO-Pink; *WAPAL* – CYMO-Green; *PTEN* – CYMO-Red; and *FAS* – CYMO-Aqua. We have shown previously that use of the probes bracketing *PTEN* improves the fidelity of assessments of PTEN loss (28). The two probes *WAPAL* and *FAS* on either side of PTEN provide information about the size of larger deletions and also allow recognition of background artifactual losses of *PTEN* due to histologic sectioning. Artifacts in assessing *PTEN* loss can arise when histologic sectioning cuts away part of the nucleus containing the *PTEN* locus in cells in the section while leaving the centromere in place. The latter is a result of the long distance between the centromere and the *PTEN* locus on chromosome 10.

PTEN FISH analysis was performed entirely independently of PTEN IHC, using 5 micron TMA sections stained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) in tumor areas selected by a pathologist who was not involved in PTEN IHC scoring (TJ) using an immediately adjacent section stained with hematoxylin and eosin. *PTEN* copy number was evaluated by counting spots for all four probes using SemRock filters appropriate for the excitation and emission spectra of each dye in 50–100 non-overlapping, intact, interphase nuclei per tumor TMA core. For the initial blinded analysis of each case, two tumor-containing cores were scored based on the overall quality of FISH hybridization. In cases where different clonal deletions were present, all

three cores were analyzed and more cells were analyzed. Hemizygous (single copy) *PTEN* loss was assigned when >50% of nuclei exhibited either interstitial loss of *PTEN* or concomitant loss of adjacent genes (*PTEN* and *WAPAL* and/or *FAS*). Homozygous deletion was defined by a simultaneous lack of both *PTEN* locus signals in 30% of scored nuclei.

IHC-guided re-analysis of cases with discrepant results by IHC and FISH: 53 cases showed PTEN protein loss by IHC with 2 copies of *PTEN* gene present by initial FISH analysis (see Results, below). Two cases showed PTEN protein intact by IHC with homozygous *PTEN* deletion by *PTEN* FISH. To analyze the cause of these discrepancies, we re-examined both the IHC and FISH data in these cases. A digitally scanned photomicrograph of the most representative core with IHC loss was selected to guide FISH re-analysis of the identical core from each case. Since the majority (85%) of these discrepant cases showed only focal IHC loss in a subset of glands, the FISH re-analyses concentrated on determining the *PTEN* gene copy number within these small areas guided by the IHC staining. Since only 50-100 cells from the best two of the three tumor-containing cores were initially analyzed for each case by *PTEN* FISH (28), this more extensive analysis could include TMA cores and regions of TMA sections that had not been studied by FISH during initial blinded analysis.

Results:

Data for PTEN FISH and IHC in a subset of the CFRPTMR cohort were separately reported previously (17, 25). Briefly, of the 1275 patients with tissue sampled for the tissue microarrays (TMAs), 86% (1095/1275) had evaluable PTEN status by IHC and 14% (180/1275) had missing data. Of these, 17% (30/180) were missing due to ambiguous immunostaining results and 83% (150/180) had absence of tumor tissue present on the TMA slides. Of the tumors with evaluable staining, 24% (258/1095) showed any PTEN protein loss, with 14% (150/1095) showing heterogeneous PTEN loss (in some but not all sampled tumor glands, best exemplified by case #10 in Figure 4A), and 10% (108/1095) showing homogeneous PTEN loss (in all sampled tumor glands). The remaining 76% (837/1095) of cases had intact PTEN protein by IHC in all sampled tumor glands. *PTEN* FISH results were evaluable in 64% of the cases sampled on the TMA (810/1275). Of the evaluable cases, *PTEN* FISH showed any *PTEN* deletion in 18% of cases, with 9% (70/810) of cases showing hemizygous deletion and 9% (75/810) of cases showing homozygous *PTEN* deletion. The remaining 82% (665/810) of cases showed two intact *PTEN* alleles.

PTEN IHC results were available on 90% of cases with interpretable *PTEN* FISH results (731/810). The rates of *PTEN* gene and PTEN protein loss were quite similar in the subset with both FISH and IHC results compared to the entire evaluable cohort for each assay reported separately. Overall, 22% (158/731) of cases with interpretable IHC and FISH results showed PTEN protein loss, with 13% (96/731) showing

heterogeneous loss and 8% (62/731) showing homogeneous loss. Similarly, 17% (129/731) of cases with interpretable IHC and FISH results showed *PTEN* gene deletion (8% hemizygous and 9% homozygous).

Overall, intact PTEN IHC was 91% specific for lack of underlying *PTEN* gene deletion. Of cases with 2 copies of the *PTEN* gene by FISH analysis, 549/602 showed intact PTEN protein (**Figure 1**, **Table 1**). Notably, 85% (45/53) of the discrepant cases (loss of PTEN protein expression by IHC and 2 copies of *PTEN* gene by FISH analysis) showed heterogeneous PTEN protein loss in some, but not all, sampled tumor glands, suggesting the possibility that a small area with *PTEN* deletion may have been missed in the initial FISH analysis (see below). PTEN IHC loss was 65% sensitive for the detection of underlying hemizygous *PTEN* gene deletion since 40/62 of cases with hemizygous *PTEN* gene deletion by FISH showed PTEN protein loss by IHC (**Figure 2**). Of these cases, 65% (26/40) showed heterogeneous PTEN loss in some but not all sampled tumor glands. PTEN IHC loss was 97% sensitive for homozygous *PTEN* gene deletion. Of cases with homozygous gene deletion by FISH, 65/67 showed PTEN protein loss by IHC (**Figure 3**). Only 37% (25/67) of the cases with homozygous *PTEN* gene deletion and PTEN protein loss had heterogeneous loss of PTEN protein by IHC. The fraction of tumors with underlying homozygous *PTEN* gene deletion differed by the extent of PTEN protein loss observed: 26% (25/96) tumors with heterogeneous PTEN protein loss had an underlying homozygous *PTEN* deletion compared to 64% (40/62) of tumors with homogeneous PTEN protein loss ($p<0.0001$ by Fisher's exact test).

The negative predictive value for intact PTEN IHC was 96% (549/573) for lack of any gene deletion and 99.6% (571/573) for lack of homozygous *PTEN* deletion. The

positive predictive value of PTEN IHC loss for presence of any *PTEN* gene deletion (homozygous or hemizygous) was 66% (105/158) overall, or 53% (51/96) for heterogeneous PTEN protein loss and 87% (54/62) for homogeneous PTEN protein loss.

Next, we re-examined cases where there was a discrepancy between the PTEN IHC and FISH. Overall, 53 cases with PTEN protein loss had two intact copies of *PTEN* by FISH, of which 85% (45/53) showed heterogeneous PTEN protein loss. Since only 50-100 tumor cells from two of the three tumor cores from each cases were initially evaluated by FISH, it is possible that focal tumor areas with *PTEN* gene deletion by FISH were missed or not analyzed in this blinded analysis. To examine this and other possible explanations for the IHC-FISH discrepancy, each of these 53 discordant cases were re-reviewed for IHC and FISH staining. IHC-guided FISH re-analysis in these cases revealed borderline IHC loss in 6% (3/53) cases (**Figure 4A, Case #10**) and failure to analyze the identical tumor core or area by both IHC and FISH in 34% (18/53) cases. Of the remaining discrepant cases where the IHC result was convincing and the identical tumor area was analyzed by both methods, 41% (13/32) revealed hemizygous (n=8, **Figure 4A, Case #11**) or homozygous (n=5, **Figure 4A, Case #12**) deletion that was focal in 94% (11/13) cases and thus likely missed on initial FISH analysis. The remaining 59% (19/32) of these cases showed two copies of *PTEN*, thus representing truly discordant cases. One explanation for these cases is presence of a small deletion and/or mutation undetectable by FISH at one or both *PTEN* alleles. Another possibility is that even though the same core was evaluated by both methods in these cases, there may be heterogeneity within the core such that different levels of the core sampled on

the FISH and IHC slide may have been truly heterogeneous (**Figure 4B, Case # 13**). Of the two discrepant cases with homozygous *PTEN* deletion and intact PTEN protein, different tumor areas were analyzed in one case. In the other case, a minute focus of tumor with PTEN loss by IHC that was initially missed was observed on re-examination (**Figure 4B, Case # 14**).

Discussion:

There is an increasing need for validated prognostic and predictive biomarkers in prostate cancer at both ends of the clinical spectrum. Developing prognostic biomarkers to help select patients who are appropriate for active surveillance, as well as predictive biomarkers to guide the application of targeted therapy in metastatic disease remain major areas of unmet clinical need. PTEN has long been a promising marker in both regards, however, until relatively recently the lack of well validated antibodies to detect PTEN loss has made it challenging to incorporate into routine pathologic risk assessment protocols or clinical trials of PI3K-targeted agents in prostate cancer. Due to this difficulty, FISH has historically been used to assess whether PTEN is an effective prognostic biomarker by testing the association of *PTEN* gene deletion with prostate cancer progression. The results from these studies have consistently shown that *PTEN* gene deletion is associated with increased Gleason grade and stage in prostate cancer (6, 8, 10, 17, 29, 30). In addition, *PTEN* gene deletion is independently associated with prostate cancer progression and death (6-16). Though many of these previous studies have used 2-color FISH, there is increasing evidence that 4-color probes are better suited to distinguish true gene deletions from sectioning artifacts in interphase FISH studies (Yoshimoto et al in preparation). Accordingly, our group recently demonstrated that homozygous PTEN deletion by 4-color FISH is associated with decreased recurrence-free survival in a subset of the prostate tumor cohort examined in the current study (17).

Despite these compelling data, *PTEN* FISH has not been widely implemented in clinical prostate cancer risk stratification protocols to date for a number of reasons. First, FISH to detect gene deletions is technically challenging, requiring careful probe design (28) and rigorous cutoffs to ensure that sectioning artifacts do not result in false calls of deletion. Detecting of hemizygous deletions can be particularly challenging when nuclei are overlapping or have been distorted during preparation. Depending on tissue quality and fixation, there may also be difficulties with optimizing protease digestion such that as many as 30-40% of cases cannot be evaluated on the first attempt when using TMAs, though this may be less of an issue for biopsies (17). In large part because it is so technically challenging, FISH is relatively expensive compared to IHC, and it has been harder to integrate the daily workflow of pathology laboratories as a reflexive test. Finally, though *PTEN* is most commonly lost via larger genomic deletions in prostate cancer, as many as 10-20% of cases may have mutations, small insertions or deletions that are not detectable by FISH, in addition to potential epigenetic and miRNA-mediated mechanisms of PTEN loss (1-5, 24). To address these challenges, several groups have developed immunohistochemistry (IHC) assays to query PTEN status in tissue (9, 18, 31). While a number of such assays have been published, for the most part, these assays have largely been compared to 2-color FISH in only small scale studies with less than 100 tumors each (22, 23, 32). In the only large studies to compare IHC and FISH, there was only weak (κ=0.5) (14) or no significant correlation (13) between the assays, suggesting a failure of the IHC and/or FISH assay to analytically validate.

We used a commercially available rabbit monoclonal antibody to develop an immunohistochemistry (IHC) assay to assess PTEN protein loss in prostate cancer and showed that this assay is reasonably sensitive for detection of *PTEN* gene deletion by 2-color FISH or high density SNP array in prostate cancer samples and shows minimal inter-observer variability in interpretation (9). Similarly, the assay performed well versus 4-color FISH in a small cohort of needle biopsy specimens (27). Using this assay, our group previously demonstrated that PTEN protein loss is associated with an increased risk of recurrence and progression in surgically treated cohorts of prostate cancer patients (11, 12).

To facilitate clinical use of the assay, we adapted it to the automated Ventana staining platform with clinical-grade reagents suitable for *in vitro* diagnostic use. In the current study, we analytically validated this automated assay by comparing it to 4-color *PTEN* FISH across a large multi-institutional cohort of prostate cancer patients. Remarkably, we found that the automated IHC assay was 91% specific for 2 intact copies of the *PTEN* gene and 97% sensitive for homozygous *PTEN* gene deletions. This is by far the highest sensitivity and specificity reported for a PTEN IHC assay relative to FISH. This improved sensitivity and specificity is in part due to the improved specificity of the automated IHC assay versus the manual assay and also due to the improved 4-color FISH assay which uses two *PTEN* gene flanking probes, in addition to a centromeric control and a *PTEN* probe to detect *PTEN* gene deletions. Surprisingly, the IHC assay was also 65% sensitive for detection of hemizygous *PTEN* gene deletion, suggesting that there is complete protein loss in a large fraction, perhaps even a majority, of apparently hemizygous cases. This is most likely due to truncating

mutations (nonsense, frameshift and splice site mutations) or epigenetic modifications at the second allele that are undetectable by FISH yet lead to protein loss (1, 3, 5, 33). Interestingly, though the prevalence of such mutations in *PTEN* is below 5% in most prostate tumors, many of these mutations are truncating alterations occurring in cases with hemizygous deletions that would lead to protein loss detectable by IHC (1-5).

In addition to the potential increased sensitivity of IHC versus FISH for detecting combinations of events including copy loss, point mutations, small insertions and deletions and epigenetic modifications leading to *PTEN* inactivation, IHC is also very useful for screening for areas of focal PTEN loss. By necessity, *PTEN* FISH is analyzed at high magnification, examining 50-100 nuclei, which may miss small areas of loss within the sampled tumor. In contrast, IHC can be easily screened at low magnification and still afford a nearly cell-by-cell resolution image of PTEN expression. In the current study, in over 40% of cases where PTEN IHC detected loss and *PTEN* FISH was initially read as 2 copies in the identical tumor core, rescreening the FISH guided by areas of IHC loss resulted in detection of small areas with *PTEN* deletion, initially missed or beneath the cutoff for the FISH scoring. This result, in addition to the high negative predictive value of intact IHC for lack of deletion strongly suggests that IHC screening for PTEN loss is likely to be an efficient and cost-effective strategy to ascertain PTEN status in tissue sections.

Akin to HER2 assessment in breast, it is ultimately likely that the best protocol will be to perform reflexive FISH on a subset of prostate tumors after initial IHC screening. Clearly, in cases with ambiguous IHC results (<5%), FISH will have an important role. However, there may also be a role for FISH in cases with

heterogeneous loss of PTEN by IHC. As in previous cohorts (12), in the current cohort we found that homogeneous PTEN IHC loss was more strongly associated with decreased recurrence-free survival compared to heterogeneous PTEN protein loss in both univariate and multivariate analyses (25). The explanation for why focal PTEN loss is a less potent prognostic indicator than homogeneous loss remains unclear. Homogeneous PTEN loss may be a surrogate indicator for expansion of a single, dominant clone of tumor cells. Alternatively, perhaps loss of PTEN in a larger number of cells increases risk of tumor progression for stochastic reasons. Finally, this result may also be related to the higher prevalence of homozygous *PTEN* deletion among the cases with homogeneous IHC loss, compared to the cases with heterogeneous IHC loss (64% vs 26%; $p<0.0001$ by Fisher's exact test). Indeed, in the subset of the current cohort where *PTEN* FISH was correlated with disease outcomes, only homozygous but not hemizygous *PTEN* loss was independently associated with decreased recurrence free survival in multivariate models (17). Thus, it may be that tumors with heterogeneous PTEN protein loss and underlying homozygous *PTEN* gene deletion have outcomes roughly equivalent to cases with homogeneous PTEN protein loss (the majority of which have homozygous deletion). Though larger case numbers than were included in the current study will be required to formally address this hypothesis, this would suggest that it may useful to perform reflexive FISH in the case of heterogeneous PTEN protein loss by IHC (14% of total cases in current cohort) to determine whether there is underlying homozygous *PTEN* gene deletion. The FISH could be guided by the IHC to focus on areas with protein loss, increasing the sensitivity of the assay in this way.

There are a number of limitations of the current study. Though both FISH and IHC were performed on the same TMAs, analysis of all TMA cores was not technically feasible for both methods in all cases and correlation between the two assays was done on a tumor-by-tumor rather than core-by-core basis for most cases. Thus, some of the disagreements between FISH and IHC likely came about because of tumor heterogeneity, where different areas of the same tumor were being analyzed by each assay. In addition, the gold-standard for assessing *PTEN* gene status is not clear at this point. Though FISH can detect larger deletions which are the most common mechanism of loss in prostate cancer, it will miss smaller deletions, as well as indels and missense mutations which may inactivate the gene. Thus, in cases where the same tumor tissue was analyzed, it is impossible to know the true cause of the apparent discrepancies between FISH and IHC without using a third methodology such as sequencing to examine for gene alterations that would be missed by FISH (these studies are ongoing in separate cohorts currently). Finally, due to the relatively small numbers of discordant cases overall, it was not feasible to do a meaningful analysis comparing FISH and IHC for prediction of prognosis in these cases, to determine which assay is a better prognostic tool.

In conclusion, in a large multi-institutional cohort of prostate tumors, our IHC assay for PTEN loss shows the highest specificity and sensitivity for *PTEN* gene deletion reported for an IHC assay to date. These data strongly suggest that IHC is a cost-efficient method to screen for PTEN loss in prostate tumors, requiring ~$100 and a single 4 μm tumor section for assay performance. In cases with ambiguous PTEN IHC results or heterogeneous PTEN protein loss, reflexive *PTEN* FISH may be a useful

confirmatory test. Ultimately, screening for PTEN status is likely a useful prognostic biomarker in prostate cancer, and may prove to be predictive for response to PI3K-pathway targeted therapeutics currently in clinical trials.

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Figure Legends:

Figure 1: Prostate cancer cases showing intact PTEN protein with 2 intact PTEN gene alleles. Cases #1 and 2: PTEN immunohistochemistry (IHC) demonstrates intact PTEN protein (left), while four-color FISH image from adjacent section (right) shows two intact *PTEN* alleles (see enlarged inset—two red signals) with two intact copies flanking genes, *WAPAL* (green) and *FAS* (aqua) as well as chromosome 10 centromeres (pink).

Figure 2: Prostate cancer cases showing variable PTEN protein expression with hemizygous *PTEN* gene deletion. Case #3: PTEN immunohistochemistry (IHC) demonstrates intact PTEN protein (left), with four-color FISH image from an adjacent section showing a hemizygous *PTEN* deletion with loss of one *PTEN* gene (see enlarged inset-one red signal). Since both centromeres (pink) and the *WAPAL* (green) and *FAS* (aqua) probes that flank either side of *PTEN* are retained it is likely that this hemizygous deletion is interstitial and restricted to the *PTEN* region. Case #4: PTEN IHC image shows homogeneous loss of PTEN protein (left) while FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset-one red

signal). Concurrent hemizygous deletion of the adjacent *FAS* gene probe (one aqua signal missing) but retention of two copies of the centromere and *WAPAL* gene probes indicates the deletion includes both the *PTEN* and *FAS* genes. Case #5: PTEN IHC image shows somewhat light, but intact immunostaining for PTEN protein (left) while the FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset-one red signal). Since there was concurrent loss of the *WAPAL, PTEN* and *FAS* gene probes (green, red and aqua, respectively), but retention of both centromeres (pink), this hemizygous deletion extends outside the *PTEN* region in both directions.

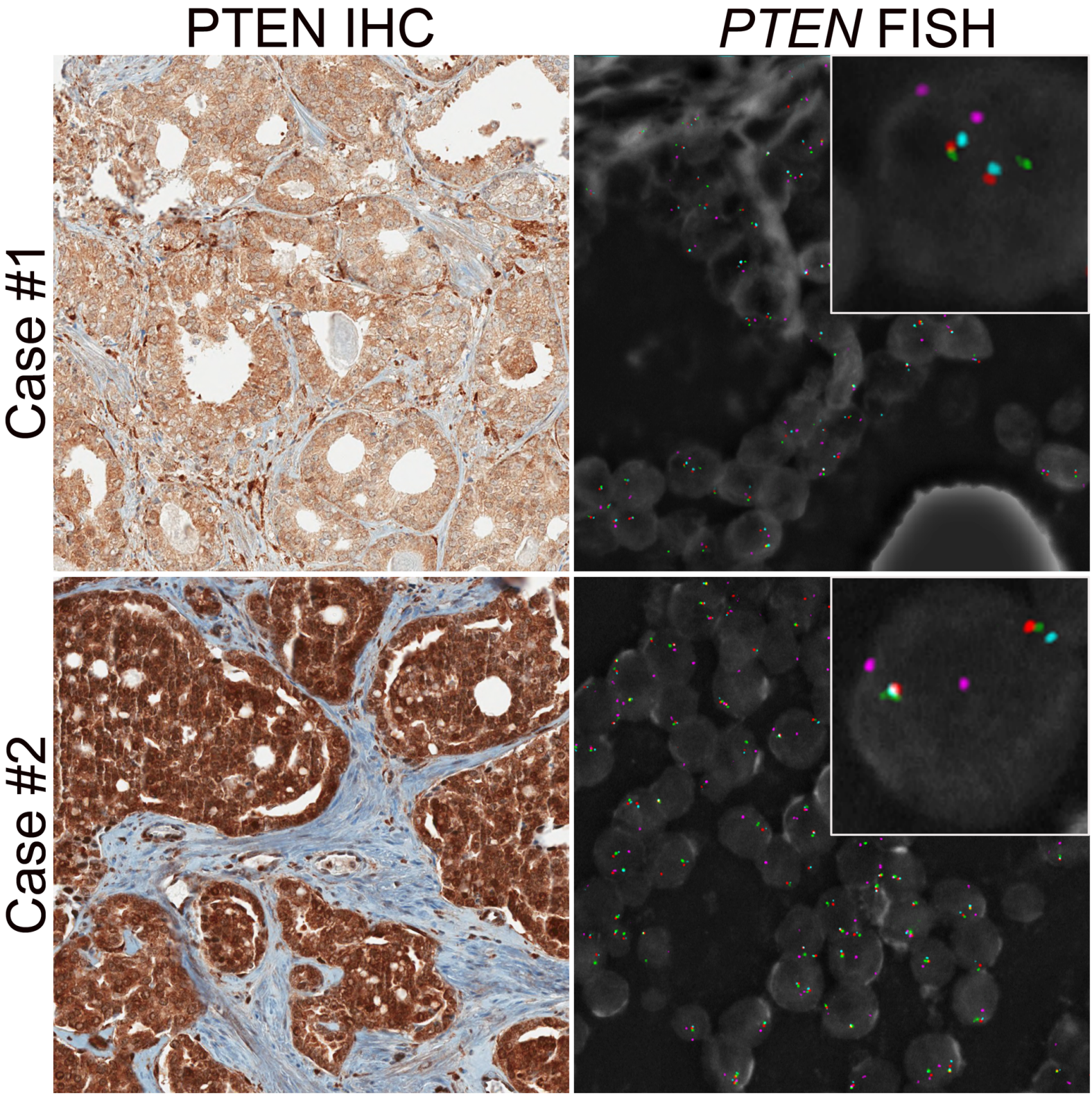
Figure 3: Prostate cancer cases showing absence of PTEN protein expression with homozygous *PTEN* gene deletion. Case #6: PTEN immunohistochemistry (IHC) image (left) shows loss of PTEN protein in tumor glands. Intraductal spread of tumor is present in this case and retention of PTEN protein is seen in benign basal and luminal cells of duct containing tumor (arrowhead). Four-color FISH image from an adjacent section (right) shows a homozygous deletion with loss of both *PTEN* genes (see enlarged inset - no red signals). The retention of the centromeres (pink) and both *WAPAL* genes (green), but the presence of only one copy of the *FAS* gene (aqua) indicates that one of the deletions involved both the *PTEN* and *FAS* genes. Case #7: PTEN IHC image (left) shows loss of PTEN protein in tumor glands, with retention in entrapped benign gland (B). FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset - no red signals). The retention of the centromeres (pink) but concurrent loss of one *WAPAL* (green) and one *FAS* gene (blue) indicates the deletions extend outside the *PTEN* region. Case #8: PTEN IHC image

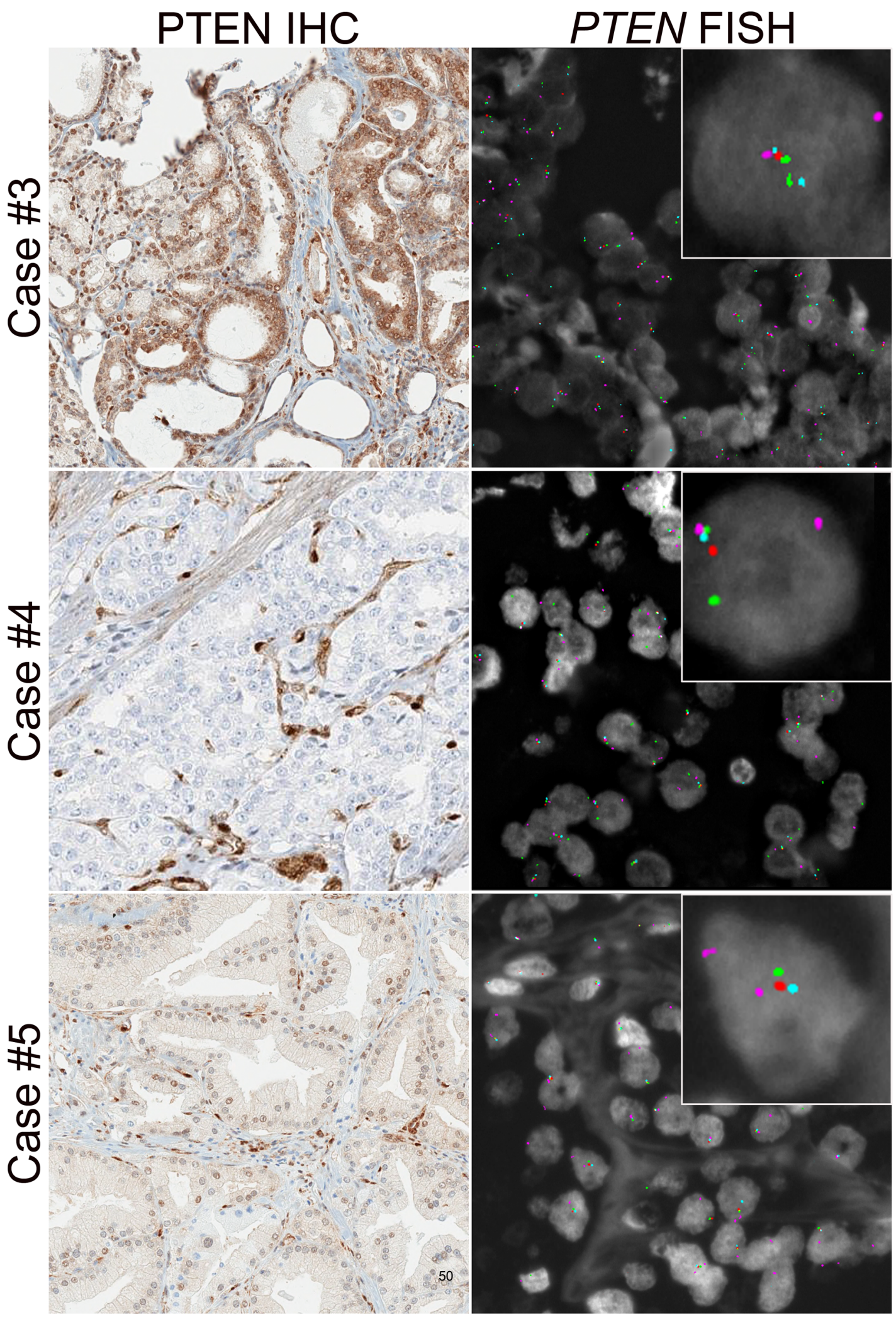
(left) shows loss of PTEN protein in tumor glands, with retention in adjacent benign gland (B) and nearby endothelial cells (arrowhead). **(**FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset - no red signals). The retention of the centromeres and both the *WAPAL* genes (green), but the concurrent loss of both *FAS* (blue) and *PTEN* (red) indicates that both copies of chromosomes 10 have deletions involving these genes.

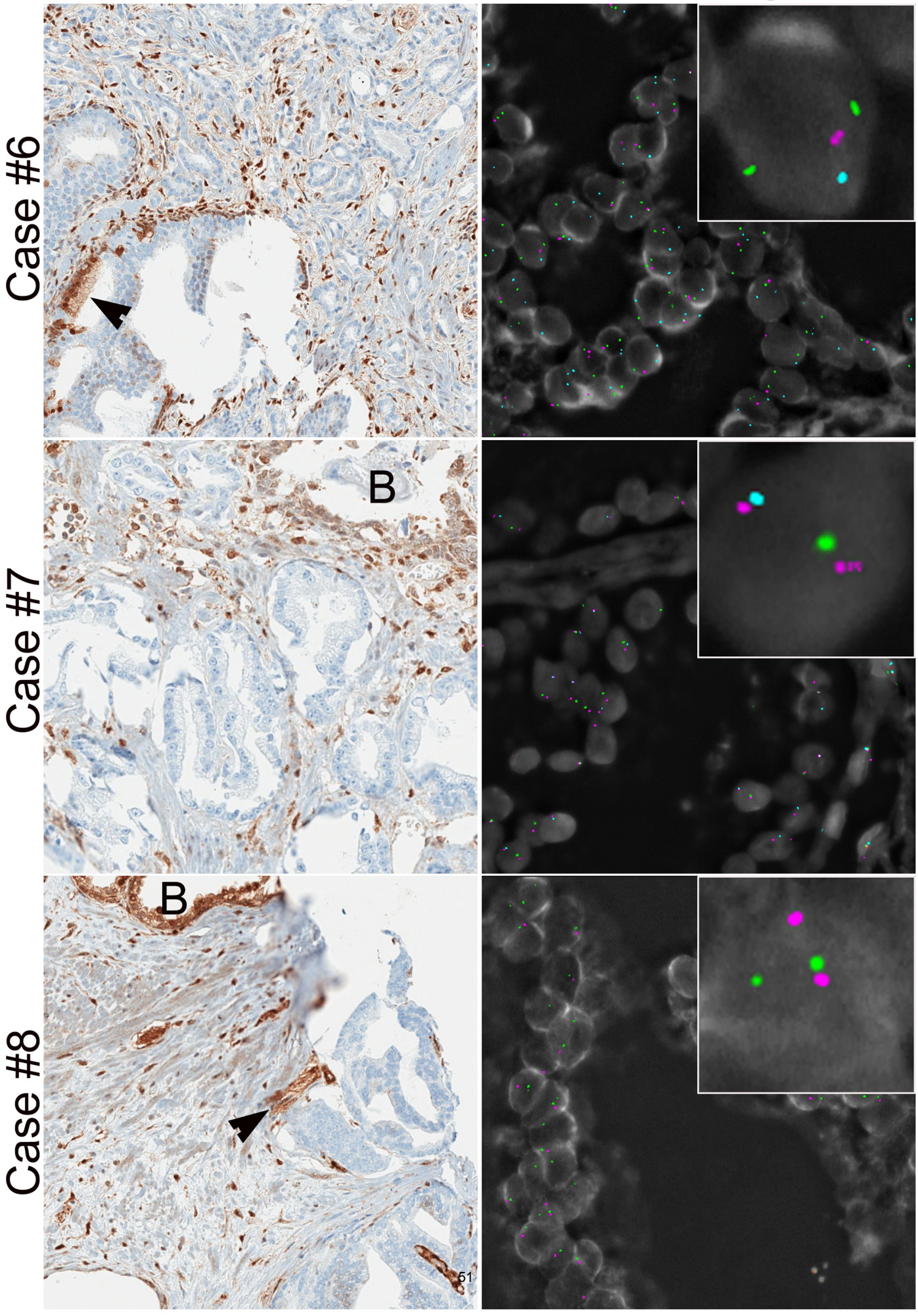
Figure 4A: Prostate cancer cases with discordant PTEN immunohistochemistry (IHC) and FISH results on initial review. Case #9: PTEN immunohistochemistry demonstrates very weak cytoplasmic immunostaining with loss of nuclear immunostaining and thus was called negative on initial review, though in retrospect it may be better classified as ambiguous due to weak staining and absence of benign glands for comparison (left). Four-color FISH image from an adjacent section that is representative of all examined cores in this TMA (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN* and *FAS* gene probes are each present as two copies. Case #10: PTEN IHC image (left) shows heterogeneous PTEN loss in some tumor glands (arrow) but PTEN protein is expressed by majority of other tumor glands in this core. FISH image from an adjacent section (right) was initially read as PTEN intact, but shows a focal area with hemizygous *PTEN* deletion recognized on re-examination guided by IHC. The enlarged inset shows there is only one copy of the red *PTEN* gene probe (one red signal) and loss of both aqua *FAS* gene probes. Case #11: PTEN IHC image (left) demonstrates heterogeneous PTEN loss in some tumors glands (arrows) but not in others

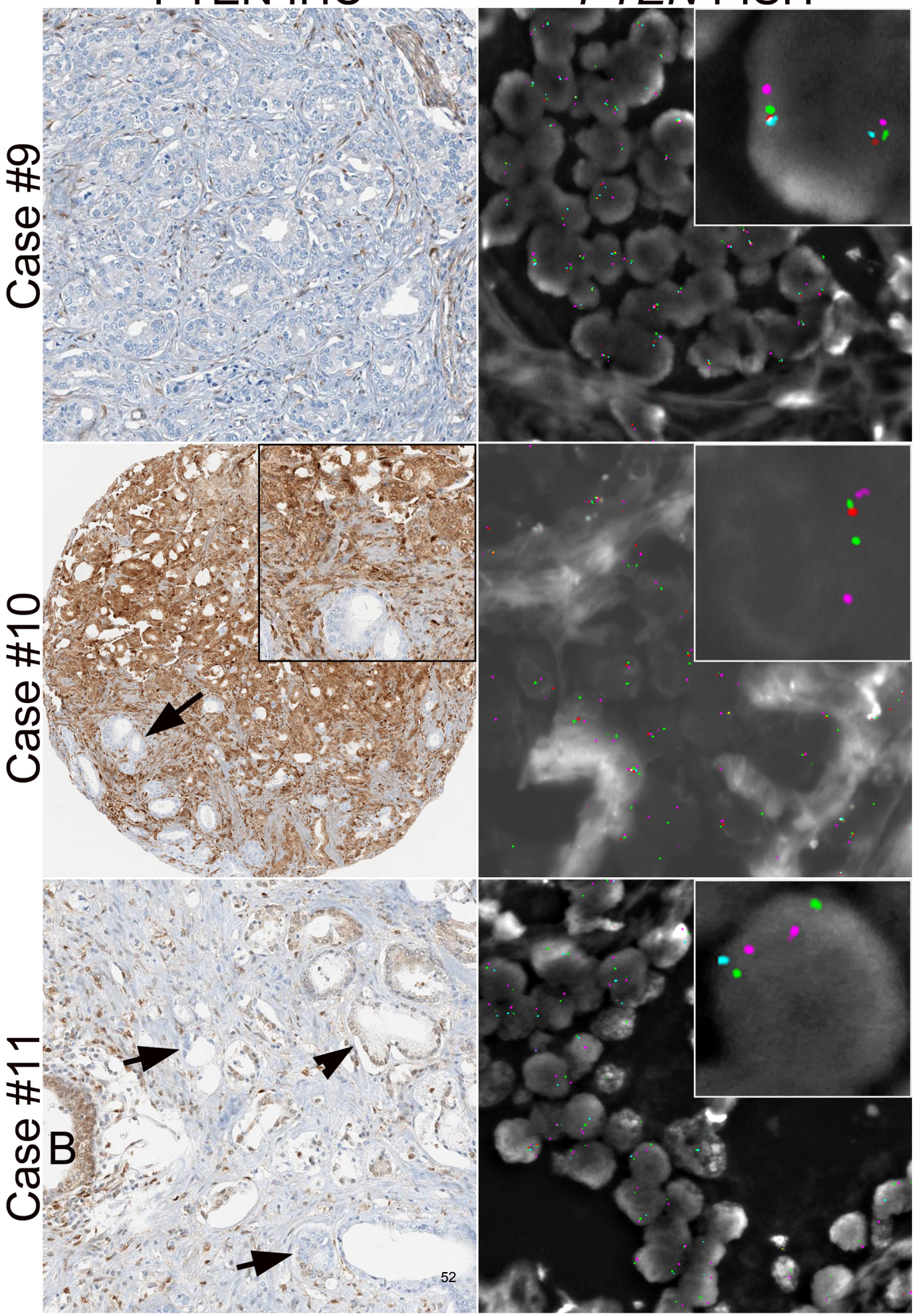
(arrowheads). FISH image from an adjacent section (right) shows the small area of the section that had a homozygous *PTEN* deletion on re-examination. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and one copy of the aqua *FAS* gene probe, but retention of the adjacent *WAPAL* and centromere probes.

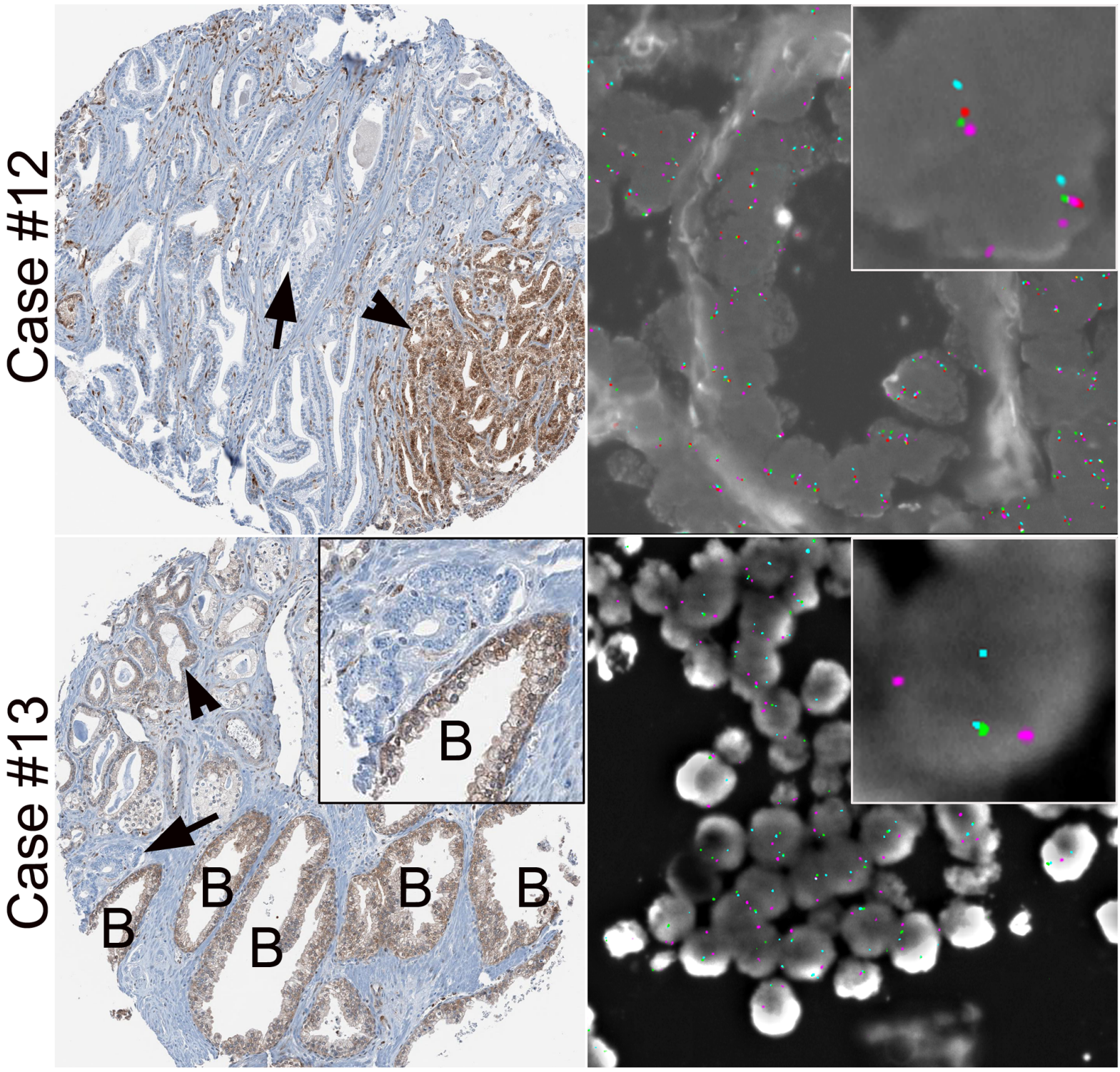
Figure 4B: Prostate cancer cases with discordant PTEN immunohistochemistry (IHC) and FISH results on initial review. Case #12: PTEN immunohistochemistry (IHC) image (left) shows heterogeneous loss of PTEN protein in some tumor glands (arrow) but not in others (arrowhead). A FISH image from an adjacent section that is representative of all examined cores in this TMA (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN* and *FAS* gene probes are each present as two copies. The heterogeneous loss in this case may have resulted in different tumor areas sampled in slides for IHC and that for FISH. Case #13: PTEN immunohistochemistry (IHC) image (left) shows predominantly intact/light immunostaining in tumor glands (arrowhead) and benign glands (B) with a very focal area of tumor with PTEN loss identified on re-review after FISH analysis (arrowhead, inset). FISH analysis of an adjacent section to the IHC indicates a homozygous *PTEN* deletion. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and loss of one green *WAPAL* gene probe but retention of both the *FAS* and the centromere probes.


PTEN IHC
PTEN FISH
Case #1
Case #2


PTEN IHC
PTEN FISH
Case #3
Case #4
Case #5
50


PTEN IHC
PTEN FISH
Case #6
Case #7
Case #8
B
B


PTEN IHC
PTEN FISH
Case #9
Case #10
Case #11
B


PTEN IHC
PTEN FISH
Case #12
Case #13
B
B
B
B
B
B

Summary of PTEN IHC by PTEN FISH status.						
	PTEN FISH					
	Intact		Hemi-deletion		Homo-deletion	
	N	%	N	%	N	%
PTEN IHC						
Intact	549	91	22	35	2	3
Heterogeneous loss	45	7	26	42	25	37
Homogeneous loss	8	1	14	23	40	60

12. FIGURES AND TABLES:

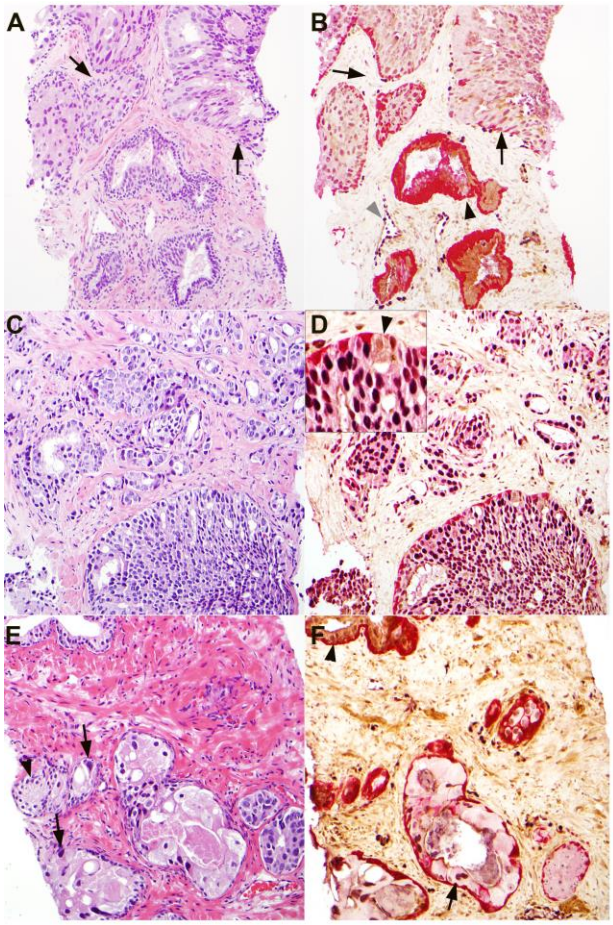


Figure 1: PTEN loss and ERG expression are common in morphologically diagnosed intraductal carcinoma of the prostate on needle biopsy (A) Isolated intraductal carcinoma case (arrows). (B) Quadruple immunostain for PTEN (brown), ERG (purple) and basal cells (red) on case in (A) demonstrates PTEN loss in intraductal carcinoma (arrow) compared to nearby benign gland (arrowhead). ERG is expressed. (C) Intraductal carcinoma with nearby invasive carcinoma. (D) Quadruple immunostain for PTEN (brown), ERG (purple) and basal cells (red) on case in (C) demonstrates PTEN loss and ERG expression in intraductal carcinoma cells (inset) relative to entrapped benign cells (inset, arrowhead). (E) Intraductal carcinoma with marked cytological atypia. (F) Quadruple immunostain for PTEN (brown), ERG (purple) and basal cells (red) on case in (E) demonstrates PTEN loss in intraductal carcinoma cells (arrow) relative to nearby benign glands (arrowhead). ERG is also expressed in this case.

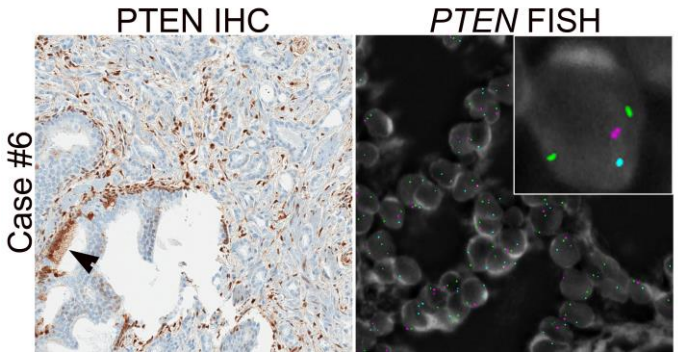


Figure 2: Prostate cancer case showing absence of PTEN protein expression with homozygous *PTEN* gene deletion. PTEN immunohistochemistry (IHC) image (left) shows loss of PTEN protein in tumor glands. Intraductal spread of tumor is present in this case and retention of PTEN protein is seen in benign basal and luminal cells of duct containing tumor (arrowhead). Four-color FISH image from an adjacent section (right) shows a homozygous deletion with loss of both *PTEN* genes (see enlarged inset - no red signals). The retention of the centromeres (pink) and both *WAPAL* genes (green), but the presence of only one copy of the *FAS* gene (aqua) indicates that one of the deletions involved both the *PTEN* and *FAS* genes.

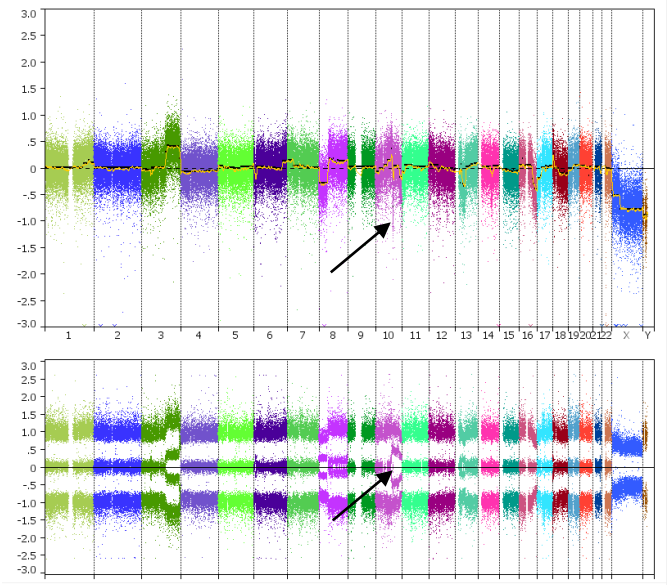


Figure 3: Oncoscan whole genome copy number derived from FFPE tissue. This case contained a focal homozygous deletion at *PTEN* on 10q (arrow), which was confirmed by IHC (not shown). This methodology requires only 80 ng of input DNA, optimal for small intraductal lesions.

Table 1: Nucleic acid quantity and quality metrics comparing two isolation methods (QIAmp vs AllPrep) in 3 cases of FFPE prostate cancer using two tumor macrodissection methods (sections vs cores)

Case		QIAamp DNA FFPE Kit		Qiagen AllPrep Kit				
		DNA [ ] (ng/ul)	Total DNA (ug)	DNA [ ] (ng/ul)	Total DNA (ug)	RNA [ ] (ng/ul)	RNA RIN Number	DV200 (%)
8284	Sections	223.0	11.2	226.0	9.0	35068	2.2	97
	Cores	29.2	1.5	26.4	1.1	88	2.5	92
	Homogenized Cores	-	-	44.0	1.8	81	2.6	10
57571	Sections	108.0	5.4	106.0	4.2	113	5.1	93
	Cores	49.5	2.5	57.4	2.3	296	1.3	96
	Homogenized Cores	-	-	132.0	5.3	229	2.5	26
14839	Sections	102.0	5.1	97.9	3.9	195	5.2	97
	Cores	84.0	4.2	79.1	3.2	5932	2.3	97
	Homogenized Cores	-	-	82.4	3.296	38	1.3	39